

МІНІСТЕРСТВО ОСВІТИ І НАУКИ УКРАЇНИ
КИЇВСЬКИЙ НАЦІОНАЛЬНИЙ УНІВЕРСИТЕТ ІМЕНІ ТАРАСА ШЕВЧЕНКА

МОТУЗЮК ОЛЕКСАНДР ПЕТРОВИЧ

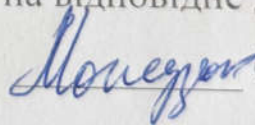
УДК 612.741.1:616.748-092:615.015.2:547.279

ДИСЕРТАЦІЯ

**Скоротлива активність скелетних м'язів за хронічної алкогольної
міопатії та її корекція водорозчинними C_{60} фулеренами**

Подається на здобуття наукового ступеня доктора біологічних наук

Дисертація містить результати власних досліджень. Використання ідей, результатів і текстів інших авторів мають посилання на відповідне джерело

 О.П. Мотузюк

Науковий консультант: доктор фізико-математичних наук, професор
Прилуцький Юрій Іванович

АНОТАЦІЯ

Мотузок О. П. Скоротлива активність скелетних м'язів за хронічної алкогольної міопатії та її корекція водорозчинними C_{60} фулеренами.

Дисертація на здобуття наукового ступеня доктора біологічних наук за спеціальністю 03.00.02 - біофізика – Київський національний університет імені Тараса Шевченка, Київ, 2026.

За використання тензометрії, біохімічного та гістологічного аналізів досліджено скоротливу активність скелетних м'язів (швидких і повільних) щурів за хронічної алкогольної міопатії та її корекцію водорозчинними C_{60} фулеренами залежно від дози, схеми (до вживання алкоголю, вживання разом з акоголем і після вживання алкоголю) та перорального застосування.

Уперше на моделях алкоголізованих тварин доведено, що застосування водорозчинних C_{60} фулеренів у терапевтичній схемі введення (пероральна щоденна доза 1 мг/кг разом з алкоголем упродовж 3, 6 і 9 місяців), яка виявилася найбільш ефективною, нормалізує час встановлення гладкого тетануса м'язів, збільшує їх силову відповідь, зменшує «тремор» та подовжує час настання м'язової втоми порівняно з контролем. Біохімічні дослідження крові та м'язової тканини піддослідних тварин засвідчили відновлення показників про- та антиоксидантного балансу та ключових ферментів енергетичного обміну. Гістологічні дослідження підтвердили зниження дистрофічних і некротичних змін у м'язових волокнах за використання водорозчинних C_{60} фулеренів разом з алкоголем. Також виявлено зменшення рівня ферментів-маркерів ураження печінки порівняно з контролем. У печінці спостерігали слабо виражену дрібнокраплинну ліпідну дистрофію гепатоцитів, балонна дистрофія була відсутня, що свідчить про гепатопротекторну дію водорозчинних C_{60} фулеренів.

Уперше в рамках модельних розрахунків показано, що наночастинки C_{60} фулеренів здатні ефективно зв'язувати молекули етанолу, виключаючи їх з метаболічного циклу, і, таким чином, зменшувати негативний вплив алкоголю на організм загалом і скоротливу активність скелетних м'язів зокрема.

Одержані результати свідчать про перспективність використання

водорозчинних C_{60} фулеренів-антиоксидантів у терапії алкогольної міопатії та пов'язаних з нею уражень печінки.

Ключові слова: скелетні м'язи; алкогольна інтоксикація; хронічна алкогольна міопатія; біомеханічні параметри скорочення м'яза; біохімічний аналіз; гістологічний аналіз; водорозчинний C_{60} фулерен; антиоксидантна дія; моделювання

SUMMARY

Motuziuk O. P. Contractile activity of skeletal muscles in chronic alcoholic myopathy and its correction by water-soluble C_{60} fullerenes.

Dissertation for the scientific degree of Doctor of Biological Sciences in speciality 03.00.02 - biophysics – Taras Shevchenko National University of Kyiv, Kyiv, 2026.

Using tensometry, biochemical and histological analyses, the contractile activity of skeletal muscles (fast and slow) in rats with chronic alcoholic myopathy and its correction by water-soluble C_{60} fullerenes depending on the dose, regimen (before alcohol consumption, consumption together with alcohol and after alcohol consumption) and oral administration were investigated.

For the first time, it has been proven in models of alcoholized animals that the use of water-soluble C_{60} fullerenes in the therapeutic administration regimen (oral daily dose of 1 mg/kg together with alcohol for 3, 6 and 9 months), which turned out to be the most effective, normalizes the time of establishment of smooth muscle tetanus, increases their force response, reduces "tremor" and prolongs the time of onset of muscle fatigue compared to the control. Biochemical studies of the blood and muscle tissue of experimental animals showed the restoration of pro- and antioxidant balance indicators and key enzymes of energy metabolism. Histological studies confirmed the reduction of dystrophic and necrotic changes in muscle fibers with the use of water-soluble C_{60} fullerenes together with alcohol. A decrease in the level of enzymes-markers of liver damage compared to the control was also found. In the liver, mildly expressed small-droplet lipid dystrophy of hepatocytes was observed, balloon dystrophy was absent, which indicates the hepatoprotective effect of water-soluble C_{60} fullerenes.

For the first time, within a calculation model it was shown that C_{60} fullerene

nanoparticles are able to effectively bind ethanol molecules, excluding them from the metabolic cycle, and thus reduce the negative impact of alcohol on the body in general and the contractile activity of skeletal muscles in particular.

The results obtained indicate the prospects for the use of water-soluble C₆₀ fullerene-antioxidants in the therapy of alcoholic myopathy and associated liver lesions.

Keywords: skeletal muscles; alcohol intoxication; chronic alcoholic myopathy; biomechanical parameters of muscle contraction; biochemical analysis; histological analysis; water-soluble C₆₀ fullerene; antioxidant action; modeling.

ЗМІСТ

АНОТАЦІЯ.....	2
ПЕРЕЛІК УМОВНИХ СКОРОЧЕНЬ.....	6
ВСТУП.....	7
МАТЕРІАЛИ ТА МЕТОДИ ДОСЛІДЖЕННЯ.....	13
ОСНОВНІ РЕЗУЛЬТАТИ ДОСЛІДЖЕНЬ ТА ЇХ ОБГОВОРЕННЯ	
РОЗДІЛ 1. Біомеханічні параметри скорочення скелетних м'язів щурів, біохімічні показники крові та м'язової тканини, гістологічні зміни у ній за хронічної алкогольної міопатії та дії водорозчинних C ₆₀ фулеренів.....	17
РОЗДІЛ 2. C ₆₀ фулерен знижує рівень флуктуаційних коливань сили скорочення <i>musculus gastrocnemius</i> за хронічної алкогольної міопатії	30
РОЗДІЛ 3. Вплив водорозчинних C ₆₀ фулеренів на розвиток патологічних процесів у печінці хронічно алкоголізованих щурів.....	35
РОЗДІЛ 4. Механізми дії водорозчинних C ₆₀ фулеренів за хронічної алкогольної міопатії.....	42
УЗАГАЛЬНЕННЯ.....	48
ВИСНОВКИ.....	52
СПИСОК ОПУБЛІКОВАНИХ ПРАЦЬ ЗА ТЕМОЮ ДИСЕРТАЦІЇ.....	54
ДОДАДОК. ОСНОВНІ ПУБЛІКАЦІЇ ЗА ТЕМОЮ ДИСЕРТАЦІЇ.....	64

ПЕРЕЛІК УМОВНИХ СКОРОЧЕНЬ

Скорочення	Розшифрування
АЛТ	аланінамінотрансфераза
АСМ	атомно-силова мікроскопія
АСТ	аспартатамінотрансфераза
АФК	активні форми кисню
АЦП	аналого-цифровий перетворювач
ГГТ	гамма-глутамілтрансфераза
ГП	глутатіонпероксидаза
ДРС	динамічне розсіювання світла
КДТ	карбогідрат-дефіцитний трансфе- рин
КФК	креатинфосфокіназа
ЛА	лактат
ЛДГ	лактатдегідрогеназа
ЛФ	лужна фосфатаза
МД	молекулярна динаміка
ПОЛ	перекисне окиснення ліпідів
C ₆₀ ВРФ	водний розчин C ₆₀ фулерена
СОД	супероксиддисмутаза
ЦАП	цифро-аналоговий перетворювач
ЦНС	центральна нервова система

ВСТУП

Актуальність дослідження. Хронічний алкоголізм - захворювання, що характеризується синдромом алкогольної залежності, внаслідок якого настають специфічні соматичні, міопатичні та нервові розлади. Смертність від алкоголізму та пов'язаних з ним супутніх захворювань посідає третє місце у світі, поступаючись лише смертності від серцево-судинних захворювань і злоякісних новоутворень [Volkow, et al., 2021]. Згідно даних ВООЗ внаслідок вживання алкоголю щорічно помирає понад 3 мільйона осіб, серед яких молоді люди віком 20-40 років складають близько 25% [Rehm, et al., 2009]. Алкогольну міопатію розглядають як самостійну патологічну форму, яка розвивається незалежно від інших проявів алкогольної хвороби, таких як ураження печінки, серцевого м'яза або алкогольної поліневропатії [Predy, et al., 2001]. Розрізняють кілька клінічних форм алкогольної міопатії, що розвиваються на тлі інтоксикації алкоголем та об'єднуються єдиним терміном – «алкоголь-індуковане ураження м'язів» [Fernández-Solà, et al., 1996]. Найпоширенішою формою алкоголь-індукованого ураження м'язів є хронічна алкогольна міопатія, яка зустрічається у 40-60% пацієнтів, які зловживають алкоголем [Vary, et al., 2008]. Хронічна алкогольна міопатія пов'язана із сукупністю складних біохімічних, фізіологічних і структурних змін скелетного м'яза, які виникають внаслідок тривалої алкогольної інтоксикації і призводять до втрати сили скорочення м'яза і його маси. Хронічна алкогольна міопатія проявляється гострою слабкістю у м'язах тонічними спазмами, високим рівнем тремору та міоглобінурією з високим ризиком розвитку гострої ниркової недостатності та летального результату. При патоморфологічному дослідженні м'язів за хронічної алкогольної міопатії виявляють гострий м'язовий некроз (рабдоміоліз) з деструкцією м'язових волокон і внутрішньоклітинним набряком, денерваційну спонтанну активність у вигляді потенціалів фібриляцій та зменшення амплітуди і тривалості потенціалів дії рухових одиниць [Bourgeois, et al., 2022].

Діагностика хронічної алкогольної міопатії пов'язана з низкою проблем. По-перше, хронічна форма алкогольної міопатії не супроводжується суттєвим підвищенням рівня креатинфосфокінази у крові; по-друге, при проведенні

електроміографії не виявляють характерних для первинного ураження м'язів патологічних змін [Simon, et al., 2023]. Показано, що морфологічною основою хронічної алкогольної міопатії є атрофія м'язових волокон за відсутності ознак фіброзу, некрозу та запальної інфільтрації, а ступінь тяжкості атрофії пов'язаний з тривалістю вживання алкоголю і практично не залежить від його дози [Singh, et al., 2000]. Прогресуюча атрофія м'язових волокон веде до втрати майже 30% маси м'яза. Встановлено, що навіть через 5 років після припинення вживання алкоголю не відбувається регресу клінічних і морфологічних ознак захворювання [Moser, et al., 2022].

Розвиток алкогольної міопатії частково пояснюють зміненими окисно-відновними станами та дисбалансом ендогенних антиоксидантів в організмі, що зумовлює відмінність між катаболізмом і синтезом білка [Otis, et al., 2009]. Алкоголь безпосередньо сприяє утворенню активних форм кисню (АФК), а його розпад у печінці призводить до утворення молекул, подальший метаболізм яких продукує АФК. Алкоголь стимулює активність цитохромів P₄₅₀, які також сприяють утворенню АФК. Таким чином, утворення АФК і окисний стрес у гепатоцитах відіграють одну з ключових ролей у розвитку алкогольної хвороби печінки [Wu, et al., 2003].

Ефективна інактивація АФК водорозчинними наночастинками C₆₀ фулерену, як потужними антиоксидантами, у системах *in vivo* [Goodarzi, et al., 2017; Gaur, et al., 2021] відкриває новий напрямок для їх біомедичного застосування, зокрема у терапії захворювань, опосередкованих окисним стресом. Важливо зазначити, що мітохондрії є основною органелою продукування АФК у скелетних м'язах за розвитку алкогольної міопатії, а відтак можна припустити, що біобезпечні (принаймні за низьких діючих доз [Prylutska, et al., 2019]) водорозчинні C₆₀ фулерени з активною мішенню на мітохондрії [Foley, et al., 2002] здатні зменшувати негативну дію алкоголю на функціонування м'язової системи.

Зв'язок роботи з науковими програмами, планами, темами. Дисертаційну роботу виконано в межах науково-дослідної тематики кафедри біофізики та нейробіології «Біофізичні ефекти і механізми дії електромагнітних полів, наноматеріалів та фармакологічних речовин на біологічні об'єкти» (№ державної

реєстрації 0106U005751), проєктів науково-технічної (експериментальної) розробки МОН України «Розробка технології застосування водорозчинних C_{60} фулеренів для зменшення втоми скелетних м'язів» (№ державної реєстрації 0121U109986) та «Інноваційна нанобіотехнологія для прискорення посттравматичного відновлення функціональної активності опорно-рухового апарату» (№ державної реєстрації 0124U001303), а також наукового дослідження і розробки за рахунок грантової підтримки конкурсу «Наука для відбудови України у воєнний та повоєнний періоди», проєкт НФДУ «Розробка технології застосування водорозчинних C_{60} фулеренів для відновлення функціональної активності скелетних м'язів на тлі механічної травми» ((№ державної реєстрації 0123U103585).

Мета та завдання дослідження: метою роботи було проаналізувати скоротливу активність скелетних м'язів за хронічної алкогольної міопатії та її корекцію водорозчинними C_{60} фулеренами.

Для досягнення цієї мети були сформульовані такі основні завдання:

1. дослідити біомеханічні параметри скорочення скелетних м'язів (швидкі та повільні) щурів після їх алкоголізації упродовж 3, 6 і 9 місяців за дії водорозчинних C_{60} фулеренів залежно від дози, схеми (до вживання алкоголю, вживання разом з акоголем і після вживання алкоголю) та перорального застосування;
2. дослідити біохімічні показники крові та м'язової тканини, гістологічні зміни у м'язах щурів після їх алкоголізації упродовж 3, 6 і 9 місяців за дії водорозчинних C_{60} фулеренів залежно від дози, схеми та перорального застосування;
3. оцінити здатність водорозчинних C_{60} фулеренів зменшувати тяжкість перебігу патологічних процесів у печінці щурів після їх алкоголізації упродовж 3, 6 і 9 місяців залежно від дози, схеми та перорального застосування;
4. дослідити механізми дії водорозчинних C_{60} фулеренів за хронічної алкогольної міопатії.

Об'єкт дослідження – скоротлива активність скелетних м'язів.

Предмет дослідження - біомеханічні параметри скорочення скелетних м'язів та біохімічні показники крові і м'язової тканини, гістологічні зміни у м'язах щурів за хронічної алкогольної міопатії та дії водорозчинних C_{60} фулеренів залежно від дози,

схеми та перорального застосування.

Методи дослідження - моделювання алкогольної міопатії *in vivo*; тензометричний аналіз скоротливої активності скелетних м'язів; світлова та атомно-силова мікроскопії; динамічне розсіювання світла; молекулярна динаміка; біохімічний аналіз; гістологічний аналіз; статистичний аналіз.

Наукова новизна одержаних результатів. Результати дослідження поглиблюють фундаментальні знання щодо розуміння скоротливої активності скелетних м'язів за розвитку хронічної алкогольної міопатії та її корекції водорозчинними C_{60} фулеренами залежно від дози, способу та схеми застосування. Зокрема, вперше запропонована терапевтична схема введення водорозчинних C_{60} фулеренів (щоденна пероральна доза 1 мг/кг разом з алкоголем упродовж експеримента) покращує біомеханічні параметри скорочення скелетних м'язів щурів на тлі їх тривалої алкоголізації, що підтверджується даними біохімічного та гістологічного аналізів. Вперше встановлено, що C_{60} фулерен знижує рівень флуктуаційних коливань сили скорочення м'язів (так званий «тремор») та подовжує час до настання їх втоми за хронічної алкогольної міопатії, зменшує тяжкість перебігу патологічних процесів у печінці щурів на тлі їх тривалої алкогольної інтоксикації, що свідчить про його гепатопротекторну дію. За використання модельних розрахунків вперше показано, що наночастинки C_{60} фулеренів здатні ефективно зв'язувати молекули етанолу, зменшуючи таким чином їх негативний вплив на функціонування м'язової системи.

Практичне значення одержаних результатів. Продемонстровані на моделях *in vivo* ефекти дії водорозчинних C_{60} фулеренів у різних часових і дозових діапазонах відкривають нові можливості для створення ефективних лікарських засобів на їх основі, що сприятиме зниженню окисного стресу у клітинах і субклітинних структурах організму та зменшенню патологічного впливу вільного етанолу на скелетно-м'язову систему.

Особистий внесок здобувача. Дисертація є самостійною науковою працею, де висвітлено власні ідеї автора, які дозволили вирішити поставлені завдання. Робота містить теоретичні та методичні положення і висновки, сформульовані здобувачем

особисто. Використані у дисертації ідеї та гіпотези інших авторів мають відповідні посилання.

Апробація результатів дисертації. Основні результати роботи доповідалися на: IV науково-практичній конференції „Актуальні питання патології за умов дії надзвичайних факторів на організм” (Тернопіль, 2013); V Всеукраїнській науково-практичній конференції „Біологічні дослідження” (Житомир, 2014 і 2015); I Всеукраїнській науково-практичній конференції з міжнародною участю «Сучасні проблеми викладання та наукових досліджень біології у ВНЗ України» (Дніпропетровськ, 2014); XI міжнародній конференції “Молодь і поступ біології” (Львів, 2015); XI Міжнародній конференції «Біологія: від молекули до біосфери» (Харків, 2016); XXI міжнародному медичному конгресі (Тернопіль, 2017); XI Міжнародній науково-практичній конференції «Молода наука Волині: пріоритети та перспективи досліджень» (Луцьк, 2017); Науково-практичній конференції «Фізіологія – медицині, фармації та педагогіці: актуальні проблеми та сучасні досягнення» (Харків, 2017); VII конгресі Наукового товариства анатомів, гістологів, ембріологів, топографоанатомів України (Одеса, 2019); Науково-практичній конференції “Прикладні аспекти морфології експериментальних і клінічних досліджень” (Тернопіль, 2019); Міжнародних наукових конференціях "Nanotechnologies and Nanomaterials" (Львів, 2022, Буковель, 2023, Ужгород, 2024, Буковель, 2025); VII, VIII та IX з'їздах Українського біофізичного товариства (Луцьк, 2015, Київ, 2019 і 2023), а також наукових семінарах ННЦ "Інститут біології та медицини" Київського національного університету імені Тараса Шевченка та кафедри фізіології людини і тварин ВНУ імені Лесі Українки.

Дисертація на здобуття наукового ступеня кандидата біологічних наук «Особливості електричної активності кори головного мозку при творчій літературній діяльності» була захищена 6 червня 2006 р. за спеціальністю 03.00.13 – фізіологія людини і тварин. Матеріали і висновки кандидатської дисертації не використані в жодній частині докторської дисертації автора.

Апробація матеріалів дисертації. За матеріалами дисертації опубліковано 10 статей у наукових фахових виданнях України та 1 статтю в іноземному фаховому виданні, 17 статей у періодичних наукових виданнях, проіндексованих у базі даних

Scopus, з них 10 статей у виданнях, віднесених до першого і другого квартилів (Q1 і Q2) відповідно до класифікації SCImago Journal and Country Rank, а також 20 тез доповідей на вітчизняних і міжнародних конференціях.

Структура та обсяг дисертації. Дисертаційна робота у вигляді наукової доповіді викладена на 63 сторінках, складається зі вступу, чотирьох розділів, узагальнення, висновків і додатка.

ОСНОВНИЙ ЗМІСТ РОБОТИ

МАТЕРІАЛИ ТА МЕТОДИ ДОСЛІДЖЕННЯ

Усі експерименти проводили на лабораторних тваринах з дотриманням міжнародних принципів «Європейської конвенції про захист хребетних тварин, які використовуються в експериментальних та інших наукових цілях» (Страсбург, 1986), та статті 26 Закону України «Про захист тварин від жорстокого поводження» (№3447-IV, 21.02.2006), а також усіх загальноприйнятих норм біоетики та біологічної безпеки. Протокол дослідження був затверджений Комісією з біоетики Київського національного університету імені Тараса Шевченка (протокол №4 від 30 липня 2024 року).

В експериментах використовували щурів-самців лінії Wistar віком від 1 місяця вагою 170 ± 5 г ($n=12$ у кожній піддослідній групі). Контрольна група отримувала 100% питну воду. Піддослідні тварини (3 групи) вживали алкоголь (40% етанол у питній воді) упродовж 3, 6 і 9 місяців. Споживання кількості етанолу розраховували у відношенні 0,5% від маси тіла тварини. Значення вмісту алкоголю у крові щурів визначали за допомогою аналізатора алкоголю (AM1, Великобританія). Перерахунок дози проводили щодобово упродовж експерименту. Інші піддослідні тварини поряд з алкоголем вживали водний розчин C_{60} фулерену (C_{60} ВРФ) у дозі 1 мг/кг ваги тварини упродовж експерименту та були рандомно поділені на такі групи: 1 – пероральне введення C_{60} ВРФ за 1 год до прийому алкоголю; 2 – пероральне введення C_{60} ВРФ разом з алкоголем; 3 – пероральне введення C_{60} ВРФ через 1 год після прийому алкоголю.

Для отримання C_{60} ВРФ використовували метод, що базується на перенесенні молекул C_{60} з толуолу у воду за ультразвукової обробки (8 Гц, 8 год) [Ritter, et al., 2015]. Отриманий C_{60} ВРФ у максимальній концентрації 0,15 мг/мл зберігає стабільність упродовж 12–18 місяців за температури $+4$ – 25°C .

Розмір наночастинок C_{60} фулерену у C_{60} ВРФ визначали методами атомно-силової мікроскопії (АСМ) та динамічного розсіювання світла (ДРС). АСМ дані (рис. 1А) демонструють присутність у воді як поодиноких молекул C_{60} (діаметр $\sim 0,7$ нм),

так і їх нанокластерів діаметром $\sim 1,4$, $\sim 2,0$, $\sim 2,8$ і $\sim 3,5$ нм.

Дані ДРС (рис. 1Б) також демонструють присутність у C_{60} ВРФ великих нанокластерів з гідродинамічним діаметром ~ 64 (8%), ~ 75 (32%), ~ 82 (42%) і ~ 106 (18%) нм. Нарешті, значення дзета-потенціалу для C_{60} ВРФ складало $-25,3$ мВ, що свідчить про негативний заряд поверхні наночастинок у розчині та його високу стабільність, тобто низьку ступінь агрегації наночастинок з часом.

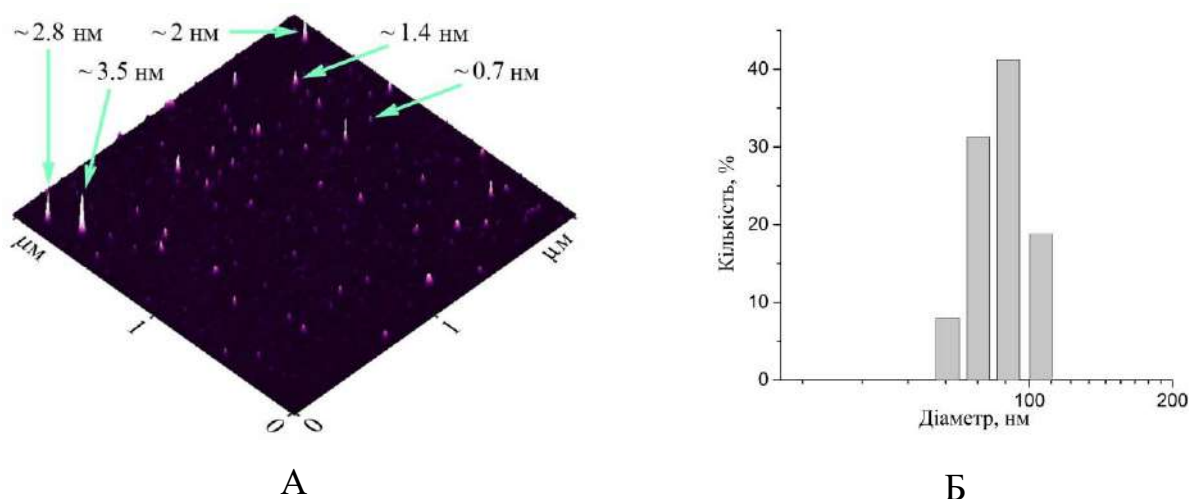


Рис. 1. АСМ зображення наночастинок C_{60} фулерену (А) та розподіл кількості наночастинок C_{60} фулерену відповідно до їх гідродинамічного діаметру (Б), присутніх у C_{60} ВРФ (0,15 мг/мл).

Для реєстрації електрофізіологічних сигналів використовували 12-ти розрядний аналого-цифровий та цифро-аналоговий перетворювач (АЦП-ЦАП, NI USB-6009, США). Вихідні імпульси ЦАП (DS2A, Digitimer, США) здійснювали стимуляцію нервів. Вхідні сигнали через підсилювач (Brownlee model 440, США) подавали на АЦП і реєстрували з частотою 10 кГц. Силу скорочення виділеного з оточуючих тканин скелетного м'яза реєстрували за допомогою тензометричного обладнання (ASI 305C-LR Dual-Mode Lever System, США). За попередньої підготовки до експерименту анестезію піддослідних тварин здійснювали внутрішньочеревним введенням золетилу (25 мг/кг, Virbac, Франція). Стандартна підготовка включала канюлювання (*a. carotis communis sinistra*) для вимірювання тиску та ламінектомію на рівні поперекового відділу спинного мозку. Для підготовки до модульованої стимуляції еферентів у

L4-L5 сегментах перерізали вентральні корінці безпосередньо у місцях їхнього виходу зі спинного мозку. Стимуляцію еферентів здійснювали електричними імпульсами тривалістю 2 мс, сформованими за допомогою генератора імпульсів. Контроль зовнішнього навантаження на скелетний м'яз здійснювали за допомогою системи механостимуляторів. Для формування стимулюючих сигналів використовували програмовані генератори сигналів спеціальної форми. Скорочення м'яза викликали безрелаксаційною електростимуляцією частотою 50 Гц упродовж 2, 4 і 6 с, а також частотами 10, 15 і 20 Гц упродовж 6 с. Для активації м'язової втоми використовували безрелаксаційну стимуляцію частотою 1 Гц тривалістю 1800 с.

При аналізі скоротливої відповіді скелетного м'яза використовували такі біомеханічні параметри, як маркери дисфункції певної ланки у ланцюзі «збудження – відповідь» м'яза, а саме: час зниження сили скорочення м'яза до нульового рівня після припинення стимуляції (t_0); час початку силової відповіді м'яза (латентний період; t_{start}); час зменшення сили скорочення м'яза на 50% від початкового рівня (t_{50}); час досягнення м'язовою силою максимального рівня (t_{max}); час встановлення гладкого тетанусу м'яза (t_1); імпульс сили м'яза (S - розрахована площа під кривою сили скорочення м'яза); мінімальна (F_{min}) та максимальна (F_{max}) сили скорочення м'яза; величина флуктуаційних коливань сили скорочення м'яза (Δf); амплітуда поодиноких скорочень м'яза (f_A).

Концентрації креатиніну, креатинфосфокінази (КФК), лактата (ЛА), лактатдегідрогенази (ЛДГ), пероксида водню (H_2O_2), відновленого глутатіона, карбогідрат-дефіцитного трансферина (КДТ), а також активності каталази, селен-залежної глутатіонпероксидази (ГП), супероксиддисмутази (СОД), аланін-аміотрансферази (АЛТ), аспартат-аміотрансферази (АСТ), гамма-глутамілтрансферази (ГГТ) та лужної фосфатази (ЛФ) у крові та м'язовій тканині піддослідних тварин, як маркери пошкодження м'язів і печінки, визначали за допомогою біохімічних аналізаторів RNL-200 та JN-1101-TR2 (Нідерланди), ABX Micros ESV60 та Pentra C400 (Франція).

Зразки скелетних м'язів та печінки фіксували у 10% формаліні і заливали у парафін. Виготовляли зрізи товщиною 5 мкм і фарбували їх гематоксиліном та еозином або гематоксиліном та пікрофуксином за методом Ван Гізона. Цифрові

мікрофотографії зразків отримували за допомогою світлового мікроскопа, суміщеного з комп'ютером (мікроскоп Olympus BX41 і цифрова фотокамера Olympus C-5050 Zoom), та проводили їх гістологічний аналіз за використання програмного забезпечення ImageJ (США).

Структура поодиноких C_{60} фулеренів була побудована за допомогою програми Avogadro [Hanwell, et al., 2012]. Нанокластери C_{60} фулеренів були побудовані за допомогою пакету програми PackMol [Martínez, et al., 2003]. Їх поміщали у розчин, що містив 1000 молекул етанолу і 23832 молекули води, що відповідає 11% спирту (модельне наближення). Молекулярна динаміка (МД) досліджуваної системи була реалізована в рамках програми NAMD [Phillips, et al., 2020] з використанням силового поля CHARMM 36 з типом TYP3P атомів води та параметрами Girifalco VDW для молекул C_{60} [Girifalco, 1991]. Тривалість МД – 30 нс за нормальних умов.

Статистичну обробку експериментальних результатів проводили за допомогою програмного пакету Statistica 8.0 (Dell, США) з використанням процедури дисперсійного аналізу (ANOVA) зі змішаним дизайном. Передбачали два міжгрупові фактори: 1) алкоголізація (три рівні – тривалість 3, 6 та 9 місяців); 2) застосування C_{60} ВРФ (два рівні – відсутність та використання C_{60} ВРФ). Припущення про те, що експериментальні дані відповідають нормальному розподілу були перевірені за допомогою тесту Шапіро-Уїлка. Для оцінки рівності дисперсій між групами використовували тест Левена. Кілька парних порівнянь між різними групами та умовами проводили за допомогою *post-hoc* тесту Бонферроні. Відмінності між групами вважали значущими за $p < 0,05$. Кожна з експериментальних силових кривих є результатом усереднення 10-ти аналогічних експериментів. Біохімічні дані представлені як середнє значення $\pm$ стандартна помилка середнього для кожної групи (повторюваність вимірювань складала не менше трьох разів).

ОСНОВНІ РЕЗУЛЬТАТИ ДОСЛІДЖЕНЬ ТА ЇХ ОБГОВОРЕННЯ

РОЗДІЛ 1. Біомеханічні параметри скорочення скелетних м'язів щурів, біохімічні показники крові та м'язової тканини, гістологічні зміни у ній за хронічної алкогольної міопатії та дії водорозчинних C₆₀ фулеренів.

Хронічна алкогольна міопатія спостерігається у понад 40% осіб, які тривалий час зловживають алкоголем, і характеризується зниженням м'язової сили, розвитком проксимального парезу та атрофією скелетних м'язів. Досі не з'ясовано, який чинник має вирішальне значення для її виникнення - тривалість вживання алкоголю, чи його кількість. Зазначимо, що ця патологія найбільш виражена у м'язах, що швидко скорочуються (*musculus gastrocnemius*). Швидкі м'язи алкоголізованих тварин розвивають значно більшу силу скорочення під час високочастотної стимуляції порівняно з повільними м'язами (*musculus soleus*). Хронічне вживання алкоголю призводить до зменшення маси *musculus gastrocnemius* та площі їх поперечного перерізу. Натомість зменшення діаметру волокон *musculus soleus* є незначним за подібних умов алкоголізації.

Етанол є токсичним агентом, що чинить як прямий, так і опосередкований вплив на м'язову систему, сприяючи розвитку алкогольної міопатії. Його метаболізм тісно пов'язаний з діяльністю ферментів, залучених до процесів окисного стресу та утворення АФК, здатних пошкоджувати клітини і тканини. Окисний стрес розвивається внаслідок порушення рівноваги між генерацією АФК та ефективністю антиоксидантного захисту. Основним шляхом метаболізму етанолу є його окиснення у печінці за участю алкогольдегідрогенази, а також цитохрому Р-450 2E1, що супроводжується утворенням АФК, порушенням редокс-балансу клітин та ушкодженням тканин різних органів.

У тварин, які вживали алкоголь упродовж тривалого часу, зафіксовано зниження активності ферментів системи глутатіону, посилення карбонілізації білків, підвищення рівнів холестеринових гідропероксидів і малондіальдегідів, що вказує на розвиток оксидативного ушкодження. У людей, які зловживають алкоголем, надмірна генерація АФК у мікросомальній та мітохондріальній системах призводить

до порушення окиснення жирних кислот і посилення перекисного окиснення ліпідів (ПОЛ) - мітохондріальні ураження виявляють майже у третини пацієнтів. Виявлений високий рівень гідропероксидів холестерину у швидкому м'язі порівняно з повільним м'язом свідчить про низьку антиоксидантну активність у м'язах, багатих на волокна типу II, за алкогольної міопатії. Ефективність антиоксидантних механізмів у збалансуванні підвищеного утворення АФК у швидких м'язах знижена, що робить їх більш чутливими до окисного стресу та, зрештою, веде до порушення скоротливої функції, швидкої втоми і травмування.

Вуглецеві наночастинки C_{60} фулеренів відомі як ефективні вловлювачі АФК у системах *in vitro* та *in vivo*. Їх антиоксидантний потенціал перевищує активність вітаміна Е у запобіганні порушенню мембранної цілісності. Встановлено, що водорозчинні C_{60} фулерени у малих дозах (0,1–1 мг/кг) зменшують м'язову втому щурів ефективніше, ніж традиційні антиоксиданти N-ацетилцистеїн і β -аланін, які широко застосовують у спортивній медицині. Вони легко (пасивна дифузія або ендцитоз) проникають всередину клітин та накопичуються переважно в мітохондріях. Встановлено, що величина LD_{50} для щурів за їх перорального введення становить 600 мг/кг. C_{60} фулерени накопичуються переважно в печінці, селезінці та шлунку і виводяться з організму упродовж 72 год із сечею. Згідно нещодавніх даних, водорозчинні C_{60} фулерени характеризуються тривалою кінетикою елімінації, що забезпечує їхню пролонговану (до 48 год) стимуляцію ендогенної антиоксидантної відповіді при м'язовому навантаженні.

Завданням цього етапу роботи було оцінити дію водорозчинних C_{60} фулеренів на біомеханічні показники скоротливої активності *musculus gastrocnemius* щурів із хронічною алкогольною інтоксикацією залежно від дози, схеми та перорального способу введення; проаналізувати біохімічні показники крові та м'язової тканини, як маркерів м'язового пошкодження, а також морфологічні зміни у м'язових волокнах; нарешті, порівняти ефективність дії водорозчинних C_{60} фулеренів на скоротливу активність *musculus gastrocnemius* та *musculus soleus* щурів за хронічної алкогольної міопатії.

Значення концентрації алкоголю у крові щурів після хронічного вживання етанолу коливалися від 147 ± 5 мг/дл (3 місяці вживання алкоголю) до 253 ± 10 мг/дл (9 місяців вживання алкоголю). Пероральне щоденне застосування C_{60} ВРФ упродовж експеримента за оптимальної дози 1 мг/кг в рамках терапевтичної схеми (разом з алкоголем) не призводило до статистично достовірних змін у вищевказаних значеннях концентрації алкоголю у крові щурів.

У групі тварин, що вживали алкоголь, вага тіла збільшилася зі 170 ± 5 г (контроль) до 200 ± 10 , 220 ± 10 і 250 ± 10 г упродовж 3, 6 і 9 місяців, відповідно. Нарешті, у групі тварин, що отримувала суміш алкоголю і C_{60} ВРФ (1 мг/кг), середня вага тіла не відрізнялася істотно від ваги тіла тварин, що отримували лише алкоголь (в межах статистичної похибки).

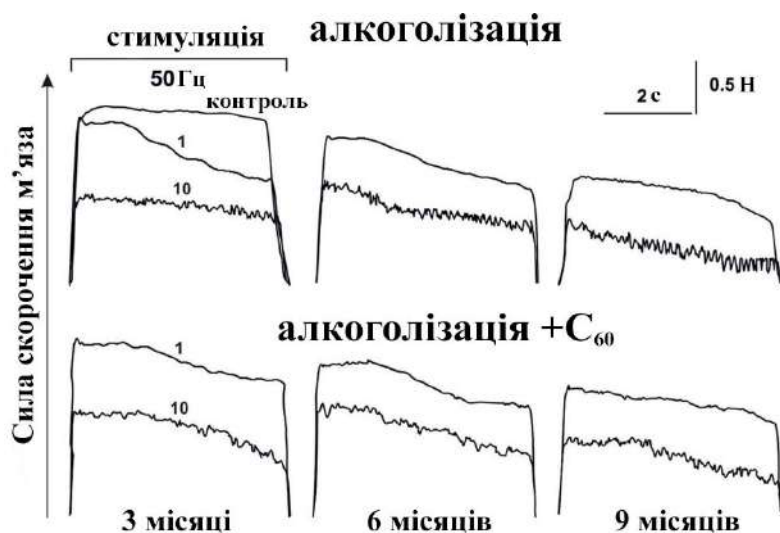


Рис. 2. Сила 1-го (1) і 10-го (10) скорочень *musculus gastrocnemius* щурів, викликаних безрелаксаційною стимуляцією частотою 50 Гц тривалістю 6 с: алкоголізація - щури, які отримували алкоголь; алкоголізація + C_{60} - щури, які отримували суміш алкоголю та C_{60} ВРФ (1 мг/кг); 3, 6 і 9 місяців - алкоголізація, що тривала 3, 6 і 9 місяців, відповідно.

М'язова слабкість є найпоширенішим клінічним проявом хронічної алкогольної міопатії, що характеризується зниженням сили м'язів та зміною часу досягнення відповідних параметрів сили скорочення, які відповідають частоті нервових імпульсів, що спостерігають у контролі. Для оцінки міопатичних порушень у *musculus*

gastrocnemius ми застосовували стимулюючий сигнал частотою 50 Гц тривалістю 6 с (рис. 2). Як бачимо, за тривалості алкоголізації 3 місяці має місце різке зниження м'язової активності вже на 1-му скороченні (на $50 \pm 3\%$) з подальшим її зниженням на 10-му скороченні на $70 \pm 3\%$ від контрольних значень. Зі збільшенням тривалості алкоголізації щурів до 6 і 9 місяців також спостерігається зменшення амплітуди скорочення *musculus gastrocnemius*. У щурів, які отримували алкоголь і C_{60} ВРФ одночасно упродовж експерименту, спостерігали підвищення м'язової сили порівняно зі щурами з відповідної групи алкоголізації.

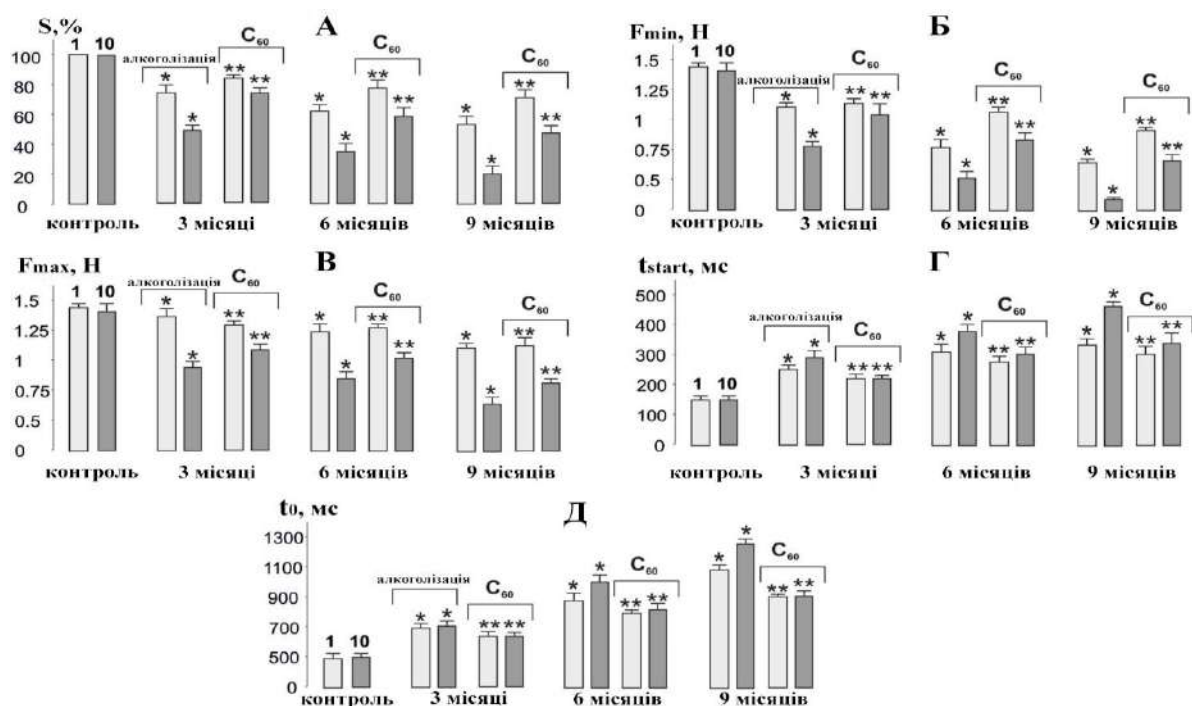


Рис. 3. Значення біомеханічних параметрів у випадку 1-го (1) і 10-го (10) скорочень *musculus gastrocnemius* щурів, викликаних безрелаксаційною стимуляцією частотою 50 Гц тривалістю 6 с: алкоголізація - щури, які отримували алкоголь; C_{60} - щури, які отримували суміш алкоголю та C_{60} ВРФ (1 мг/кг); 3, 6 і 9 місяців - алкоголізація, що тривала 3, 6 і 9 місяців, відповідно; S – імпульс сили м'яза (приймалася за 100% у випадку контролю) (А); F_{min} і F_{max} – мінімальний (Б) і максимальний (В) рівні м'язової сили; t_{start} - час початку силової відповіді м'яза (латентний період (Г)); t₀ - час зниження сили скорочення м'яза до нульового рівня після припинення стимуляції (Д); *p<0,05 відносно контролю; **p<0,05 відносно групи алкоголізації.

Імпульс сили м'яза, як показник його працездатності, є одним з найважливіших маркерів м'язової дисфункції. При 3, 6 і 9 місяцях алкоголізації він зменшився на 1-му скороченні *musculus gastrocnemius* на $22 \pm 2\%$, $29 \pm 1\%$ і $34 \pm 3\%$, відповідно. Слід зазначити, що на 10-му скороченні *musculus gastrocnemius* цей параметр знизився на $34 \pm 3\%$, $56 \pm 5\%$ і $74 \pm 4\%$, відповідно, порівняно з 1-м скороченням (рис. 3А). Застосування С₆₀ВРФ збільшило імпульс сили м'яза на $11 \pm 1\%$, $18 \pm 1\%$ і $26 \pm 2\%$ через 3, 6 та 9 місяців алкоголізації, відповідно, порівняно з групою алкоголізація. Різниця між 1-м і 10-м скороченням *musculus gastrocnemius* зменшилася на $15\text{--}39 \pm 2\%$.

Значення $F_{\min}$ є одним з найчутливіших маркерів м'язової дисфункції. Його зниження на 1-му скороченні *musculus gastrocnemius* становило $35 \pm 3\%$, $56 \pm 3\%$ і $68 \pm 5\%$ при 3, 6 і 9 місяцях алкоголізації, відповідно (рис. 3Б). На 10-му скороченні цей параметр знизився на $56 \pm 3\%$, $78 \pm 4\%$ і $91 \pm 5\%$ від контрольних значень, що свідчить про значне порушення функції м'яза. Застосування С₆₀ВРФ збільшило значення $F_{\min}$ на $10 \pm 1\%$, $26 \pm 2\%$ і $33 \pm 3\%$ при 3, 6 та 9 місяцях алкоголізації, відповідно, порівняно з групою алкоголізація.

Значення $F_{\max}$ є маркером загальної дисфункції м'язової системи, що свідчить про зниження максимально можливої силової відповіді на тлі розвитку патології (рис. 3В). На 1-му скороченні *musculus gastrocnemius* цей показник дещо знизився, а саме на $10 \pm 1\%$, $12 \pm 1\%$ і $21 \pm 2\%$ при 3, 6 і 9 місяцях алкоголізації, відповідно. Однак, на 10-му скороченні *musculus gastrocnemius* це зниження становило $47 \pm 2\%$, $52 \pm 3\%$ і $64 \pm 5\%$, відповідно, що вказує на значну дисфункцію розвитку величини $F_{\max}$. Збільшення $F_{\max}$ при застосуванні С₆₀ВРФ становило $11 \pm 1\%$, $17 \pm 1\%$ і $23 \pm 2\%$ при 3, 6 та 9 місяцях алкоголізації, відповідно, порівняно з групою алкоголізація.

Збільшення величини t_{start} свідчить про рівень фізіологічної дисфункції м'язового апарату при виконанні ним завдань, що вимагають максимальної сили під час точних позиційних рухів (рис. 3Г). Його збільшення становило $31 \pm 3\%$, $44 \pm 4\%$ і $49 \pm 4\%$ через 3, 6 і 9 місяців алкоголізації, відповідно, на 1-му скороченні *musculus gastrocnemius* з подальшим зростанням на $25\text{--}30 \pm 3\%$ на 10-му скороченні порівняно з контролем. Корекція цього параметра при застосуванні С₆₀ВРФ становила $15\text{--}20 \pm$

2% упродовж експеримента порівняно з групою алкоголізації. Слід зауважити, що у цьому випадку різниця між 1-м і 10-м скороченням *musculus gastrocnemius* не перевищувала $10 \pm 1\%$ при 9 місяцях алкоголізації.

Зростання величини t_0 свідчить про збільшення жорсткості м'яза, пов'язаної як зі збільшенням сполучнотканинних компонентів (довготривала патологія), так і змінною внутрішньом'язового тиску (гострий період патології) (рис. 3Д). Його збільшення становило $48 \pm 4\%$, $88 \pm 8\%$ і $123 \pm 8\%$ через 3, 6 і 9 місяців алкоголізації, відповідно, на 1-му скороченні *musculus gastrocnemius* з подальшим зростанням на $9-12 \pm 1\%$ на 10-му скороченні порівняно з контролем. Застосування C_{60} ВРФ зменшило цей параметр на $15 \pm 1\%$, $29 \pm 2\%$ і $41 \pm 3\%$ при 3, 6 та 9 місяцях алкоголізації, відповідно, порівняно з групою алкоголізації. Різниця між 1-м і 10-м скороченням *musculus gastrocnemius* була статистично відсутньою упродовж експерименту.

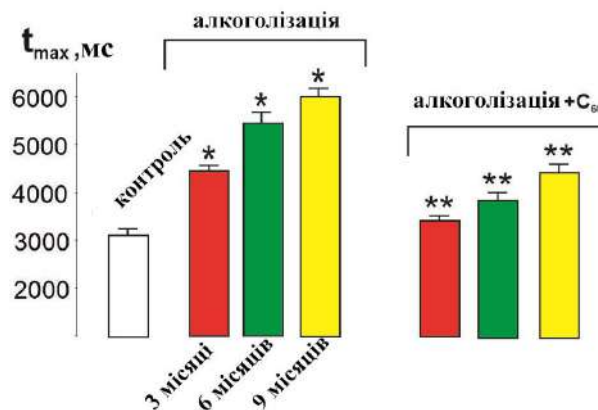


Рис. 4. Значення часу досягнення м'язовою силою максимального рівня (t_{max}) за модульованої стимуляції *musculus gastrocnemius* частотою 50 Гц тривалістю 6 с: алкоголізація - щури, які отримували алкоголь; алкоголізація + C_{60} - щури, які отримували суміш алкоголю та C_{60} ВРФ (1 мг/кг); 3, 6 і 9 місяців - алкоголізація, що тривала 3, 6 і 9 місяців, відповідно; * $p < 0,05$ відносно контролю; ** $p < 0,05$ відносно групи алкоголізації.

Час досягнення м'язовою силою максимального рівня (t_{max}) становив 4530 ± 46 , 5515 ± 52 і 6150 ± 55 мс через 3, 6 і 9 місяців алкоголізації тварин, відповідно (контроль: 3120 ± 28 мс) (рис. 4). Застосування C_{60} ВРФ зменшило цей параметр на $27 \pm 1\%$,

$30 \pm 2\%$ і $32 \pm 2\%$ при 3, 6 і 9 місяцях алкоголізації тварин, відповідно, порівняно з групою алкоголізації.

Кількісний аналіз значення часу встановлення гладкого тетанусу м'яза (t_1) може свідчити про рівень порушень в активації м'язової сили окремими руховими одиницями. Значення цього параметра становило $3,0 \pm 0,2$ с для *musculus gastrocnemius* у контролі (рис. 5). 3-х місячна алкоголізація тварин достовірно його не змінила. Водночас, алкоголізація тварин упродовж 6 і 9 місяців підвищила це значення до величин $3,2 \pm 0,2$ і $3,5 \pm 0,2$ с, відповідно. Застосування C_{60} ВРФ скоротило цей час майже до контролю.

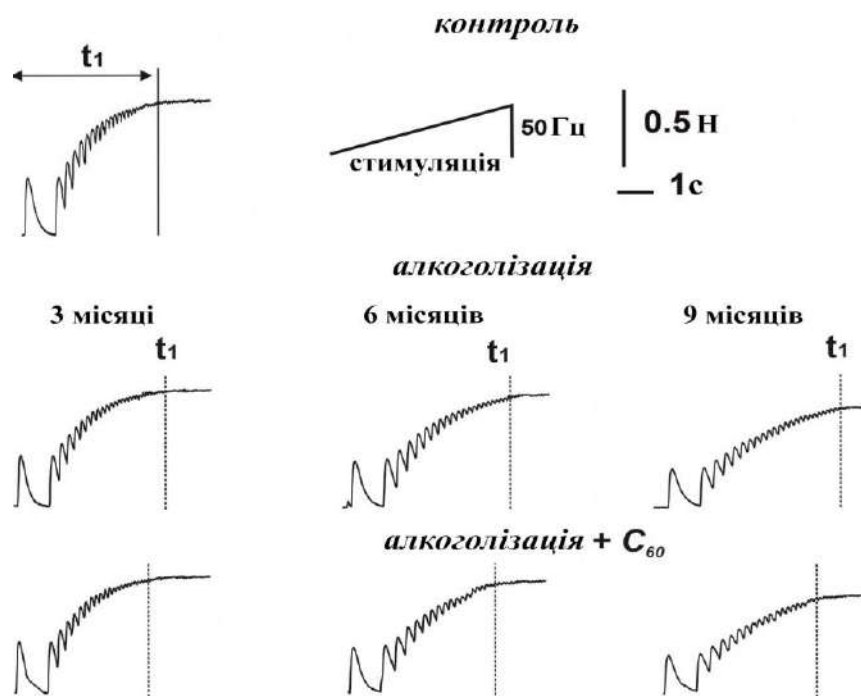


Рис. 5. Скоротлива активність *musculus gastrocnemius* щурів, викликана безрелаксаційною модульованою стимуляцією частотою 50 Гц тривалістю 6 с: алкоголізація - щури, які отримували алкоголь; алкоголізація + C_{60} - щури, які отримували суміш алкоголю та C_{60} ВРФ (1 мг/кг); 3, 6 і 9 місяців - алкоголізація, що тривала 3, 6 і 9 місяців, відповідно; t_1 - час встановлення гладкого тетанусу м'яза.

Таким чином, запропонована терапевтична схема введення C_{60} ВРФ (пероральна щоденна доза 1 мг/кг; разом з алкоголем упродовж експеримента) покращує вищезазначені біомеханічні параметри скоротливої активності *musculus gastrocnemius*

щурів на тлі їх тривалої алкоголізації, зокрема на $29 \pm 2\%$ порівняно з групою алкоголізація упродовж 9 місяців експеримента, що добре узгоджується з даними для *musculus soleus*: $25 \pm 2\%$.

Одним з ефективних підходів щодо оцінки рівня патології м'язової системи є аналіз розвитку процесів втоми під час безрелаксаційної стимуляції м'яза. Ці процеси відіграють важливу роль у забезпеченні точного позиціонування рухів скелетно-м'язового апарата. На рис. 6А показано механограми скорочення *musculus gastrocnemius* щурів за його стимуляції частотою 1 Гц упродовж 1800 с на тлі хронічної алкоголізації та дії водорозчинних C_{60} фулеренів. Як бачимо, має місце зменшення імпульса сили м'яза до $82 \pm 5\%$, $60 \pm 3\%$ і $22 \pm 1\%$ при 3, 6 і 9 місяцях алкоголізації, відповідно, порівняно з контролем (рис. 6Б). Застосування C_{60} ВРФ збільшило це значення до $94 \pm 4\%$, $85 \pm 2\%$ і $69 \pm 5\%$, відповідно.

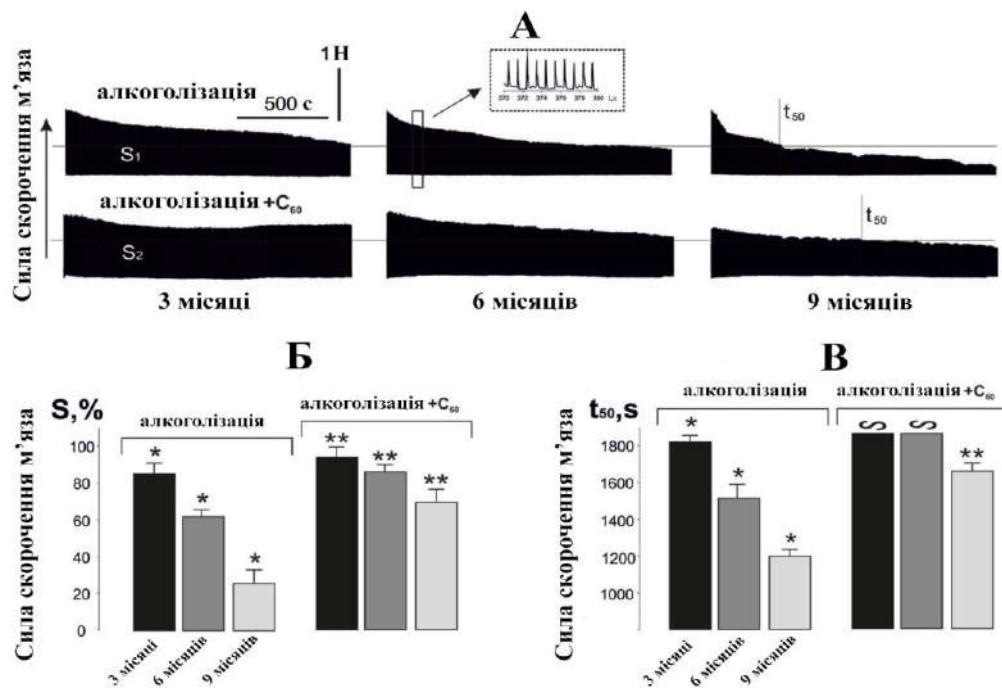


Рис. 6. Сила скорочення *musculus gastrocnemius* щурів, викликана безрелаксаційною стимуляцією частотою 1 Гц тривалістю 1800 с (А): алкоголізація - щури, які отримували алкоголь; алкоголізація + C_{60} - щури, які отримували суміш алкоголю та C_{60} ВРФ (1 мг/кг); 3, 6 і 9 місяців - алкоголізація, що тривала 3, 6 і 9 місяців, відповідно. Біомеханічні параметри розвитку м'язової втоми: S – імпульс сили м'яза (Б); t_{50} - час

зменшення сили скорочення м'яза на 50% від початкового рівня (В). $*p < 0,05$ відносно контролю; $**p < 0,05$ відносно групи алкоголізації.

Значення величини t_{50} становило 1740 ± 25 мс, 1460 ± 91 мс і 1220 ± 25 мс при 3, 6 та 9 місяцях алкоголізації, відповідно (рис. 6В). Застосування C_{60} ВРФ упродовж 3-х і 6-ти місячної алкоголізації щурів достовірно не змінило це значення. При алкоголізації, що тривала 9 місяців, цей параметр суттєво зріс порівняно з групою алкоголізації і становив 1620 ± 28 мс.

Отже, можна стверджувати, що запропонована терапевтична схема введення C_{60} ВРФ (пероральна щоденна доза 1 мг/кг; разом з алкоголем упродовж експеримента) значно (на $25 \pm 2\%$) подовжила час виникнення втоми у м'язах і є найбільш ефективною саме за тривалого розвитку алкогольної міопатії.

Отримані результати свідчать, що наслідком алкогольної міопатії є погіршення скоротливої активності м'язів. Причиною цього можуть бути як ультраструктурні зміни міоцитів та їх атрофія, так і порушення електролітного гомеостазу та біоелектричної активності загалом. За розвитку алкогольної міопатії насамперед відбувається гіперпродукція АФК у дисфункціональних мітохондріях, що веде до руйнування мембранних структур міоцитів та функціональних розладів їх ферментних систем, зокрема зниження активності Na^+/K^+ -АТФази та підвищення активності Ca^{2+} -АТФази. Такі міотичні порушення викликають дисбаланс у регуляції положення суглобів і прогресуючу м'язову слабкість, що зумовлює поступове зниження м'язової сили упродовж 3, 6 і 9 місяців алкоголізації. З функціональної точки зору це вказує на те, що пошкоджена м'язова клітина споживає більшу кількість високоенергетичних фосфатних сполук для підтримання гомеостазу. Як наслідок, відбувається порушення енергетичного метаболізму, що значно посилює м'язову втому на тлі алкогольної міопатії. Водночас, застосування водорозчинних C_{60} фулеренів сприяє підвищенню енергетичної здатності функціонуючих м'язів, уражених алкогольною інтоксикацією.

Для підтвердження одержаних біомеханічних результатів було проаналізовано біохімічні показники крові, які використовують для діагностики розвитку алкогольної міопатії. Зокрема, зміни рівнів креатиніну, КФК, ЛДГ та ЛА дозволили оцінити фізіологічний стан *musculus gastrocnemius* на тлі тривалої алкоголізації тварин (рис. 7А).

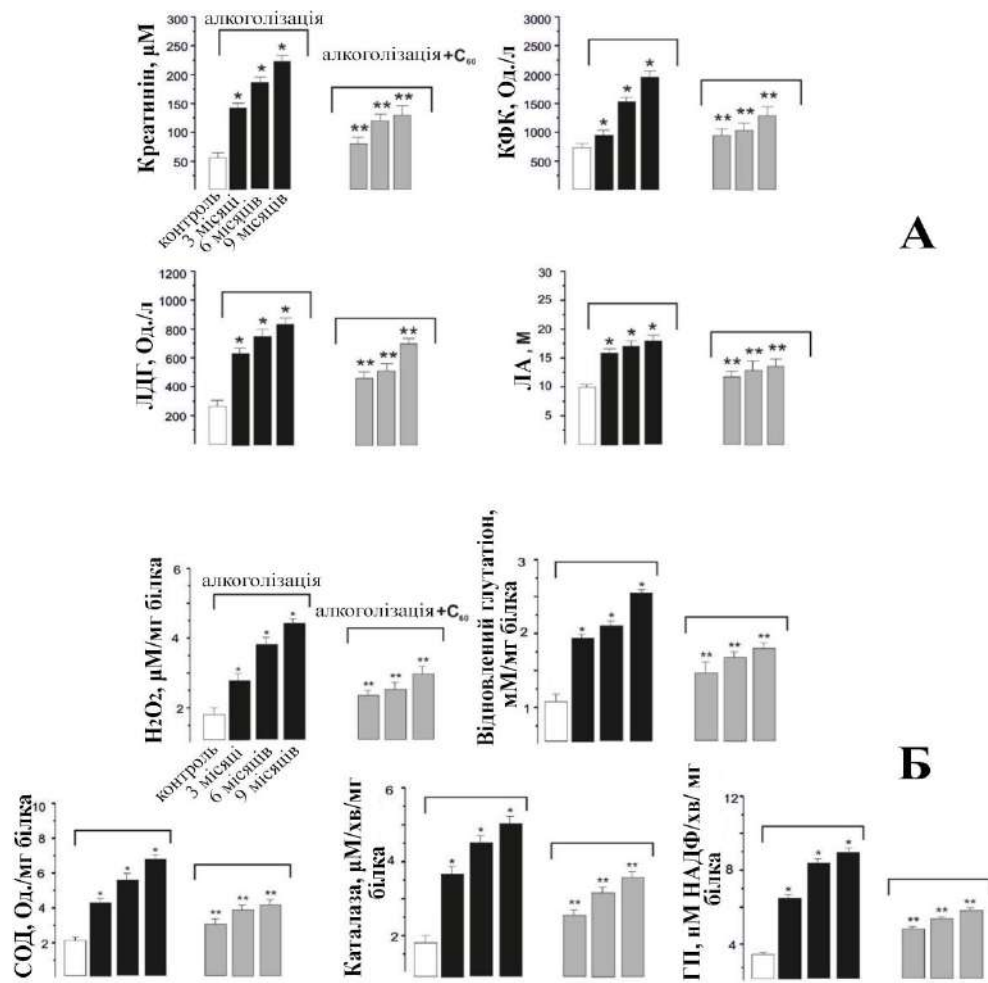


Рис. 7. Вміст креатиніну, КФК, ЛДГ та ЛА у крові піддослідних тварин (А) та показники про- та антиоксидантного балансу (H₂O₂, відновлений глутатіон, СОД, каталаза та ГП) у м'язовій тканині піддослідних тварин (Б): алкоголізація - щури, які отримували алкоголь; алкоголізація + С₆₀ - щури, які отримували суміш алкоголю та С₆₀ВРФ (1 мг/кг); 3, 6 і 9 місяців - алкоголізація, що тривала 3, 6 і 9 місяців, відповідно. * $p < 0,05$ відносно контролю; ** $p < 0,05$ відносно групи алкоголізація.

Одразу зазначимо, що вищезазначені біохімічні маркери мають виражену тенденцію до зростання зі збільшенням тривалості алкоголізації тварин, що свідчить

про суттєві пошкодження міоцитів, пов'язані насамперед з виснаженням їх енергозабезпечення та втратою іонного градієнта на мембрані. У випадках, коли щури вживали суміш алкоголю і C_{60} ВРФ, ці показники виявилися значно нижчими порівняно з тваринами, які отримували лише алкоголь. Так, креатинін знизився на $16 \pm 1\%$, $18 \pm 1\%$ і $37 \pm 2\%$, КФК - на $13 \pm 1\%$, $18 \pm 1\%$ і $28 \pm 2\%$, ЛДГ - на $15 \pm 1\%$, $29 \pm 2\%$ і $41 \pm 2\%$ та ЛА - на $19 \pm 1\%$, $27 \pm 2\%$ і $38 \pm 2\%$ при 3, 6 та 9 місяцях алкоголізації, відповідно, порівняно з групою алкоголізація (рис. 7А).

Кількісна оцінка показників про- та антиоксидантного балансу у тканинах досліджуваного м'яза є необхідною умовою для підтвердження антиоксидантної дії застосованих водорозчинних C_{60} фулеренів. Так, концентрація H_2O_2 зростала зі збільшенням тривалості алкоголізації і становила $151 \pm 7\%$, $327 \pm 9\%$ і $414 \pm 11\%$ при алкоголізації упродовж 3, 6 та 9 місяців, відповідно, порівняно з контролем. Застосування C_{60} ВРФ знижувало вміст H_2O_2 і відновленого глутатіона на $28 \pm 2\%$, $44 \pm 2\%$ та $51 \pm 2\%$ і $13 \pm 1\%$, $27 \pm 1\%$ та $42 \pm 2\%$, відповідно, активність СОД - на $21 \pm 1\%$, $29 \pm 1\%$ та $44 \pm 2\%$, активність каталази - на $12 \pm 1\%$, $34 \pm 2\%$ і $48 \pm 2\%$ та активність ГП - на $17 \pm 1\%$, $23 \pm 1\%$ і $39 \pm 2\%$ при алкоголізації упродовж 3, 6 і 9 місяців, відповідно, порівняно з групою алкоголізація (рис. 7Б). Важливо відзначити, що саме збільшення вмісту відновленого глутатіона у *musculus gastrocnemius* завдяки антиоксидантній дії водорозчинних C_{60} фулеренів сприяє підвищенню стійкості м'яза до фізичних навантажень.

Отже, у всіх проведених тестах спостерігається позитивна динаміка біохімічних показників крові та м'язової тканини піддослідних щурів за терапевтичної схеми введення C_{60} ВРФ (пероральна щоденна доза 1 мг/кг; разом з алкоголем упродовж експеримента), зокрема їх покращення становило в середньому $41 \pm 2\%$ порівняно з групою алкоголізація упродовж 9 місяців експерименту (для *musculus soleus* це становило $20 \pm 2\%$).

Важливо зазначити, що зміна досліджуваних вище біомеханічних параметрів скорочення скелетних м'язів та біохімічних показників крові та м'язової тканини піддослідних тварин на тлі їх тривалої алкогольної інтоксикації не перевищувала в середньому $10 \pm 1\%$ при збільшенні дози C_{60} ВРФ вдвічі (2 мг/кг) та суттєво зменшувалася

(на понад $25 \pm 2\%$) при зменшенні дози C_{60} ВРФ вдвічі (0,5 мг/кг) порівняно з використаною дозою C_{60} ВРФ 1 мг/кг. Нарешті, застосування C_{60} ВРФ за оптимальної дози 1 мг/кг у профілактичній (за 1 год до вживання алкоголю) і терапевтичній (через 1 год після вживання алкоголю) схемах були менш ефективні порівняно з дією C_{60} ВРФ за терапевтичної схеми (вживання разом з алкоголем).

Гістологічний аналіз продемонстрував, що у щурів, які вживали алкоголь упродовж 9 місяців (найдовший термін експеримента) (рис. 8Б і Д), більшість м'язових волокон тонші, ніж у контрольної групи (рис. 8А і Г), а середній діаметр волокон зменшується до 24,4 мкм (табл. 1). Є звивисті волокна. Деякі волокна

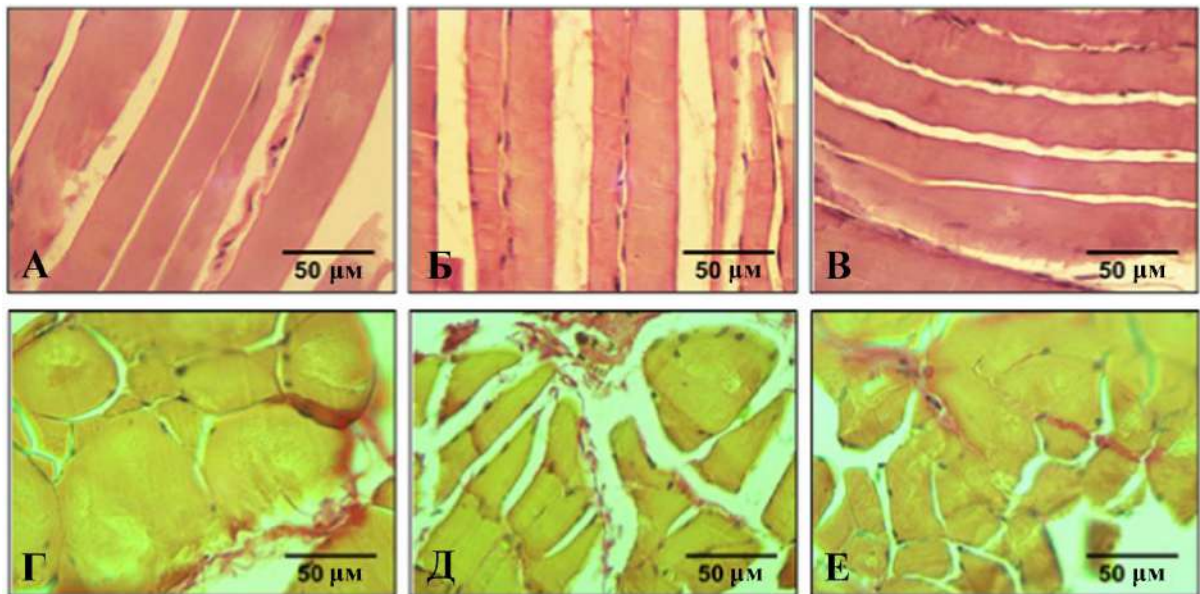


Рис. 8. Гістологічні зображення волокон *musculus gastrocnemius* піддослідних щурів: А і Г – контрольна група; Б і Д – група алкоголізація; В і Е – група алкоголізація + C_{60} . А, Б і В – фарбування гематоксилином та еозином; Г, Д і Е – фарбування гематоксилином та пікрокарфуксином за методом Ван Гізона. Масштаб: 50 μ м.

мають нерівномірну товщину по всій довжині. Деякі - вакуолізовані, що свідчить про деструктивні процеси в них. У деяких волокнах поперечна смугастість менш виражена, що може вказувати на порушення правильного просторового розташування актин-міозинових комплексів. Сполучна тканина між м'язовими волокнами збільшена (рис. 8Д), внаслідок чого площа, зайнята сполучною тканиною, збільшується до 0,21

мкм² на 1 мкм² площі поперечного перерізу м'яза (табл. 1). Відтак, спостерігається склероз і фіброз.

Таблиця 1. Морфометричні показники м'язових волокон за алкоголізації щурів та застосування C₆₀ВРФ (1 мг/кг; разом з алкоголем) упродовж 9 місяців. * $p < 0,05$ порівняно з контрольною групою; ** $p < 0,05$ порівняно з групою алкоголізація.

Група	Діаметр м'язових волокон, мкм	Площа, зайнята сполучною тканиною, мкм ² /мкм ²
Контроль	36,7 ± 1,3	0,15 ± 0,02
Алкоголізація	24,4 ± 1,4*	0,21 ± 0,02*
Алкоголізація + C ₆₀	32,8 ± 2,5**	0,17 ± 0,02**

В алкоголізованих щурів, які отримували C₆₀ВРФ, товщина м'язових волокон і площа, зайнята сполучною тканиною, незначно зменшилася і збільшилася, відповідно, порівняно з контрольною групою (табл. 1; рис. 8В і Е). Склероз і фіброз менш виражені порівняно з групою алкоголізація упродовж 9 місяців експеримента.

На основі одержаних даних можна зробити висновок, що застосування водорозчинних C₆₀ фулеренів сприяє зменшенню окислювальних процесів у м'язах шляхом підтримання балансу між прооксидантами та системою антиоксидантного захисту, запобігаючи негативному впливу АФК на клітинні та субклітинні структури за розвитку алкогольної міопатії у піддослідних тварин і, таким чином, зменшуючи деградацію м'язів.

РОЗДІЛ 2. C₆₀ фулерен знижує рівень флуктуаційних коливань сили скорочення *musculus gastrocnemius* за хронічної алкогольної міопатії.

Алкогольна міопатія є більш поширеним захворюванням, аніж, наприклад, цироз, кардіоміопатія або невропатія, спричинені алкогольною інтоксикацією організму. Тривале вживання алкоголю пригнічує синтез білка у м'язових волокнах II типу, викликає дисфункцію нервово-м'язового збудження, зменшує кількість м'язових волокон і силу м'язової відповіді. Виникнення м'язової слабкості за розвитку алкогольної міопатії певною мірою може бути компенсоване підвищеною активністю синергічних м'язових груп, що веде до уявного відчуття відсутності міотичного ураження. Однак поява значних флуктуаційних коливань сили скорочення м'яза на фазі утримання її максимальної відповіді унеможливорює корекцію точного положення суглобів. Внаслідок цього м'яз, як цілісна динамічна система, не здатний адекватно реалізувати пули нервової активності, що надходять від ЦНС. Пригнічення активності Na⁺/K⁺-АТФази зменшує специфічну провідність Na⁺, як відповідь на адекватний стимул, і, таким чином, перешкоджає розвитку збудження у міоцитах. Це може викликати тимчасовий дисбаланс у часі встановлення гладкого тетанусу м'яза алкоголізованих тварин, що, в свою чергу, збільшує рівень флуктуаційних коливань його сили скорочення (так званий «тремор»).

Завданням цього етапу роботи було оцінити дію водорозчинних C₆₀ фулеренів на величину флуктуаційних коливань сили скорочення *musculus gastrocnemius* щурів за хронічної алкогольної міопатії залежно від дози, схеми та перорального способу введення.

На рис. 9 представлено механограми 10-го скорочення *musculus gastrocnemius* піддослідних щурів за стимуляції різної тривалості: 2, 4 і 6 с. Як бачимо, стимуляційний сигнал тривалістю 2 с продемонстрував різке зниження сили м'язового скорочення на $19 \pm 1\%$, $22 \pm 2\%$ і $29 \pm 2\%$ через 3, 6 і 9 місяців алкоголізації тварин, відповідно, порівняно з контролем. Стимуляційний сигнал тривалістю 4 і 6 с зафіксував зниження сили м'язового скорочення на $22 \pm 1\%$, $35 \pm 2\%$, $41 \pm 4\%$ і $25 \pm 1\%$, $39 \pm 2\%$, $79 \pm 4\%$, відповідно, через 3, 6 і 9 місяців алкоголізації порівняно з контролем. У щурів, які перорально отримували суміш алкоголю та C₆₀ВРФ (оптимальна доза 1 мг/кг),

спостерігали збільшення м'язової сили на $14 \pm 1\%$, $18 \pm 1\%$ і $35 \pm 2\%$ через 3 місяці алкоголізації, на $19 \pm 1\%$, $28 \pm 2\%$ і $44 \pm 2\%$ через 6 місяців алкоголізації і, нарешті, на $23 \pm 1\%$, $36 \pm 2\%$ і $59 \pm 2\%$ через 9 місяців алкоголізації тварин порівняно з групою алкоголізація, коли застосовували стимуляції тривалістю 2, 4 і 6 с, відповідно.

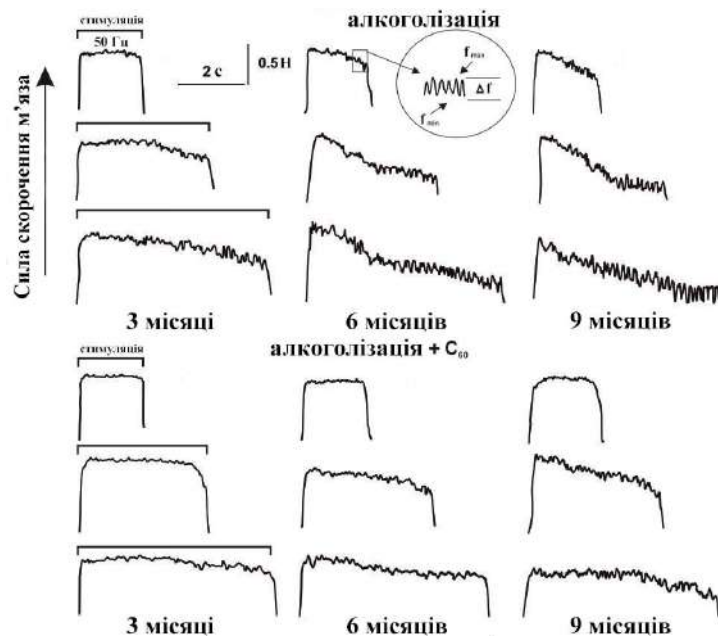


Рис. 9. Сила 10-го скорочення *musculus gastrocnemius* щурів, викликана безрелаксаційною стимуляцією частотою 50 Гц тривалістю 2, 4 і 6 с: алкоголізація - щури, які отримували алкоголь; алкоголізація + C_{60} — щури, які отримували суміш алкоголю та C_{60} ВРФ (1 мг/кг); 3, 6 і 9 місяців - алкоголізація, що тривала 3, 6 і 9 місяців, відповідно; $f_{\max}$ і $f_{\min}$ — максимальний і мінімальний рівні амплітуд флуктуаційних коливань сили скорочення *musculus gastrocnemius* на фазі утримання максимальної відповіді.

Алкоголізація упродовж 3-х місяців збільшила величину флуктуаційних коливань на фазі утримання максимальної сили скорочення м'яза з $0,030 \pm 0,005$ мН за тривалості стимуляції 2 с до $0,060 \pm 0,005$ мН за тривалості стимуляції 6 с (рис. 10). Зі збільшенням тривалості алкоголізації спостерігалось різке зростання цього параметра. Так, через 9 місяців вживання алкоголю величина флуктуаційних коливань

перевищила $0,13 \pm 0,01$ мН. У тварин, які отримували суміш алкоголю і C_{60} ВРФ, спостерігали зниження цього параметра на $53 \pm 3\%$, $56 \pm 3\%$ і $66 \pm 3\%$ через 3, 6 і 9 місяців алкоголізації, відповідно, порівняно з групою алкоголізація.

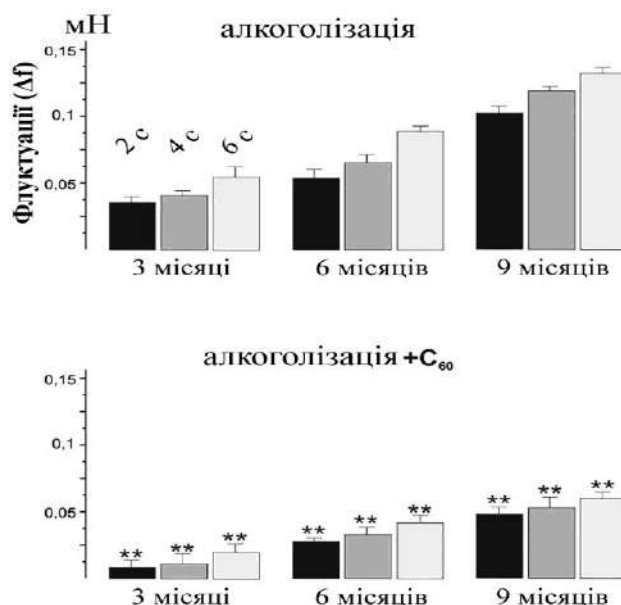


Рис. 10. Величина флуктуаційних коливань ($\Delta f = f_{\max} - f_{\min}$) сили скорочення *musculus gastrocnemius* щурів, викликана безрелаксацийною стимуляцією частотою 50 Гц тривалістю 2, 4 і 6 с: алкоголізація - щури, які отримували алкоголь; алкоголізація + C_{60} - щури, які отримували суміш алкоголю та C_{60} ВРФ (1 мг/кг); 3, 6 і 9 місяців - алкоголізація, що тривала 3, 6 і 9 місяців, відповідно. ** $p < 0,05$ відносно групи алкоголізація.

Збільшення величини флуктуаційних коливань сили скорочення *musculus gastrocnemius* алкоголізованих щурів може бути пов'язано з порушеннями процесів сумарного скорочення, подальшими часовими затримками, що перешкоджають формуванню тривалої максимальної силової відповіді м'яза. Відтак, ми детально проаналізували перехід від зубчатого до гладкого тетанусу *musculus gastrocnemius* (рис. 11).

Амплітуда поодиноких скорочень *musculus gastrocnemius* за стимуляції частотою 10 Гц збільшилася на $15 \pm 1\%$, $24 \pm 1\%$ і $56 \pm 2\%$ при алкоголізації тварин тривалістю 3, 6 і 9 місяців, відповідно, порівняно з контролем. За стимуляції частотами 15 і 20 Гц цей параметр зріс на $19 \pm 1\%$, $26 \pm 1\%$, $70 \pm 2\%$ і $27 \pm 1\%$, $48 \pm 2\%$, 80

$\pm 3\%$, відповідно. Важливо зазначити, що у цих випадках не спостерігали перехід від зубчатого до гладкого тетанусу *musculus gastrocnemius*. Застосування C_{60} ВРФ стабілізувало механограми у випадку 3-х місячної алкоголізації щурів, наблизивши їх до контролю. Зі збільшенням тривалості алкоголізації щурів до 6 і 9 місяців позитивний ефект застосування C_{60} ВРФ склав відповідно $20 \pm 1\%$, $25 \pm 2\%$, $30 \pm 2\%$ і $24 \pm 1\%$, $37 \pm 2\%$, $53 \pm 3\%$ за стимуляції частотами 10, 15 і 20 Гц, відповідно, порівняно з групою алкоголізація (рис. 11), що сприяло зменшенню флуктуаційних коливань сили скорочення *musculus gastrocnemius*.

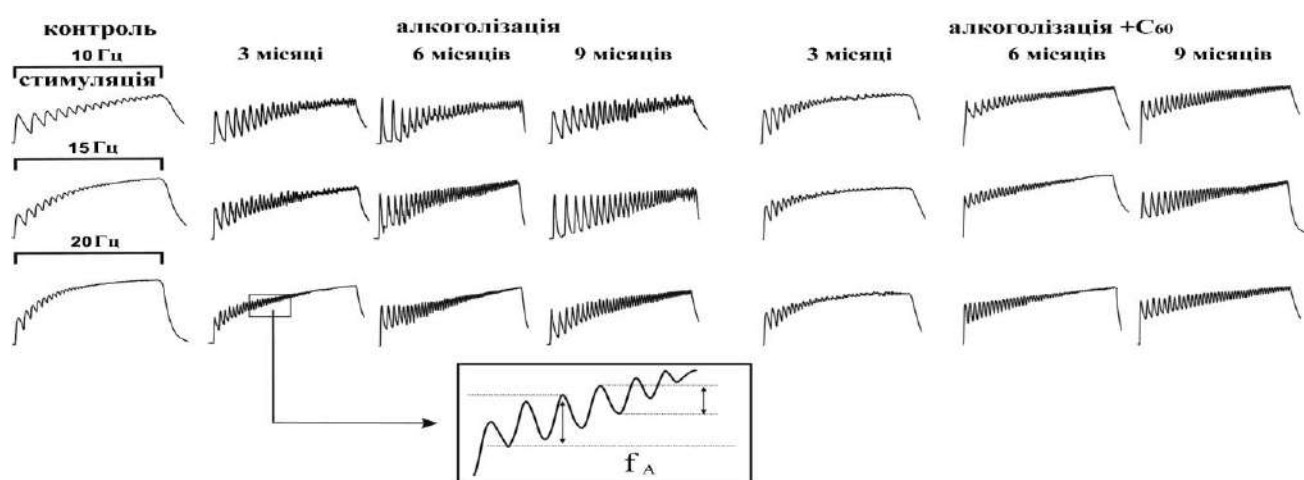


Рис. 11. Перехід від зубчатого до гладкого тетанусу *musculus gastrocnemius* при застосуванні модульованої стимуляції зі зростаючими частотами 10, 15 і 20 Гц тривалістю 6 с: алкоголізація - щури, які отримували алкоголь; алкоголізація + C_{60} - щури, які отримували суміш алкоголю та C_{60} ВРФ (1 мг/кг); 3, 6 і 9 місяців - алкоголізація, що тривала 3, 6 і 9 місяців, відповідно; f_A - амплітуда поодиноких скорочень *musculus gastrocnemius*.

Отже, можна стверджувати, що запропонована терапевтична схема введення C_{60} ВРФ (пероральна щоденна доза 1 мг/кг; разом з алкоголем упродовж експеримента) значно (на $58 \pm 3\%$) зменшує рівень флуктуаційних коливань сили скорочення *musculus gastrocnemius* на фазі утримання максимальної силової відповіді («тремор») за хронічної алкогольної міопатії.

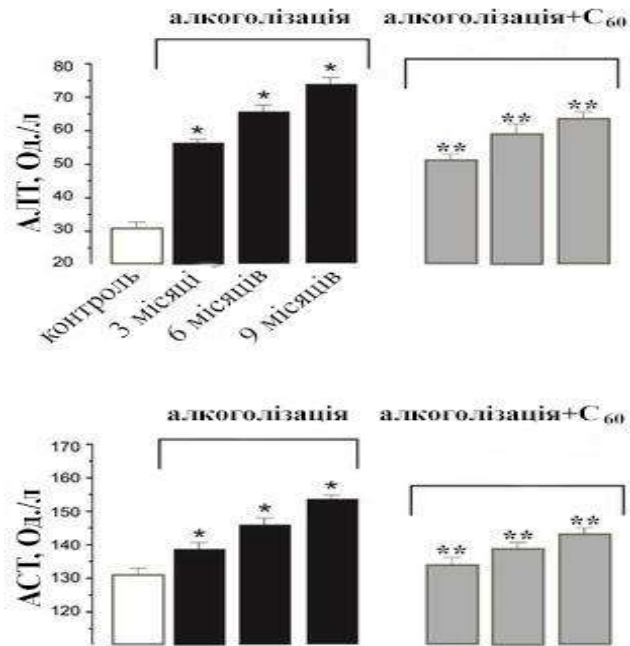


Рис. 12. Активності ферментів АЛТ і АСТ у крові піддослідних тварин: алкоголізація — щури, які отримували алкоголь; алкоголізація + C₆₀ — щури, які отримували суміш етанолу і C₆₀ВРФ (1 мг/кг); 3, 6 і 9 місяців — алкоголізація упродовж 3, 6 і 9 місяців, відповідно. * $p < 0,05$ відносно контролю; ** $p < 0,05$ відносно групи алкоголізація.

РОЗДІЛ 3. Вплив водорозчинних C₆₀ фулеренів на розвиток патологічних процесів у печінці хронічно алкоголізованих щурів.

Печінка - основний орган, відповідальний за метаболізм етанолу. Під час окислення етанолу відбувається підвищене споживання коферменту НАД⁺ та зростання співвідношення НАДН/НАД⁺. Утворений ацетальдегід впливає на продукцію цитокінів, антиоксидантних ферментів і, особливо, мітохондріального та цитозольного глутатіону. Ці сполуки здатні викликати токсичні ефекти, оскільки білки, з якими вони зв'язуються, втрачають свої функції та провокують імунну реакцію. Хронічне вживання алкоголю викликає гіпоксію у печінці, де для його метаболізму необхідний додатковий кисень. Гіпоксія сприяє утворенню АФК та продуктів ПОЛ шляхом активації ксантиноксидази. Крім того, висока концентрація алкоголю стимулює абсорбцію ендотоксинів шляхом модифікації кишкової мікрофлори та пошкодження клітин епітелію. До алкогольних захворювань печінки відносять жирову дистрофію або стеатоз, стеатогепатит, алкогольний гепатит і цироз.

Завданням цього етапу роботи було оцінити ефективність дії водорозчинних C₆₀ фулеренів на розвиток патологічних процесів у печінці щурів, спричинених хронічним алкогольним отруєнням, залежно від дози, схеми та пероального способу введення шляхом аналізу основних біохімічних показників крові, як маркерів дисфункції печінки, та гістологічних і морфометричних особливостей її пошкодження.

За хронічного вживання алкоголю підвищене накопичення продуктів ПОЛ, АФК, глікозильованих білків і вуглеводів, викликаючи окисний стрес, може бути причиною цитолізу гепатоцитів, основними біохімічними маркерами якого є активності ферментів АЛТ, АСТ і ЛФ.

Як бачимо, активність АЛТ збільшилася із 31 ± 1 Од./л у контролі до 56 ± 2 , 67 ± 3 та 74 ± 3 Од./л за алкоголізації тварин тривалістю 3, 6 та 9 місяців, відповідно (рис. 12), що свідчить про значне руйнування гепатоцитів. Застосування суміші алкоголю та C₆₀ВРФ (1 мг/кг) знизило активність цього фермента на $11 \pm 1\%$, $16 \pm 1\%$ та $18 \pm 1\%$ за алкоголізації упродовж 3, 6 та 9 місяців, відповідно, порівняно з групою алкоголізація.

Збільшення активності АСТ із 132 ± 1 Од./л у контролі до 138 ± 6 , 142 ± 7 і 154 ± 6 Од./л за алкоголізації тривалістю 3, 6 і 9 місяців, відповідно (рис. 12). Застосування С₆₀ВРФ знизило активність цього фермента в середньому на $10 \pm 1\%$ упродовж експеримента порівняно з групою алкоголізації.

У клінічній практиці для діагностики захворювань печінки використовують співвідношення активності АСТ до АЛТ у крові: коефіцієнт де Рітіса = АСТ/АЛТ. Обидва ці ферменти мають органоспецифічність, зокрема у випадку ушкодження печінки внаслідок алкогольної інтоксикації має місце підвищення активності АЛТ у крові.

Зниження коефіцієнта де Рітіса з $0,97 \pm 0,05$ у контролі до $0,80 \pm 0,07$, $0,68 \pm 0,07$ і $0,78 \pm 0,07$ через 3, 6 і 9 місяців алкоголізації тварин свідчить про прогресуюче порушення функції гепатоцитів. Застосування С₆₀ВРФ збільшило значення цього параметра в середньому на $14 \pm 1\%$ упродовж експеримента порівняно з групою алкоголізації (активність АЛТ знижується сильніше, ніж активність АСТ).

На відміну від інших ферментів печінки, продукція ГГТ «запускається» алкоголем і тому його активність може бути підвищеною у зловживаючих алкоголем пацієнтів навіть за відсутності захворювань печінки. Він є більш чутливим до порушень у клітинах печінки, аніж ферменти АЛТ і АСТ.

Як бачимо, активність ГГТ зросла із $1,6 \pm 0,1$ Од./л у контролі до $3,4 \pm 0,1$, $4,4 \pm 0,1$ та $5,9 \pm 0,2$ Од./л за алкоголізації тварин тривалістю 3, 6 та 9 місяців, відповідно (рис. 13). Застосування С₆₀ВРФ знизило активність цього фермента на $15 \pm 1\%$, $18 \pm 1\%$ та $22 \pm 1\%$ за алкоголізації тварин упродовж 3, 6 та 9 місяців, відповідно, порівняно з групою алкоголізації.

Активність фермента ЛФ у крові підвищується внаслідок захворювань печінки, зокрема гепатиту, спричиненого алкогольною інтоксикацією. Як бачимо, активність ЛФ збільшилася з $8,8 \pm 0,5$ Од./л у контролі до $9,4 \pm 0,7$, $9,8 \pm 0,7$ та $10,3 \pm 0,7$ Од./л за алкоголізації тварин тривалістю 3, 6 та 9 місяців, відповідно (рис. 13). Застосування С₆₀ВРФ зменшило активність цього фермента в середньому на $10 \pm 1\%$ упродовж експеримента порівняно з групою алкоголізації.

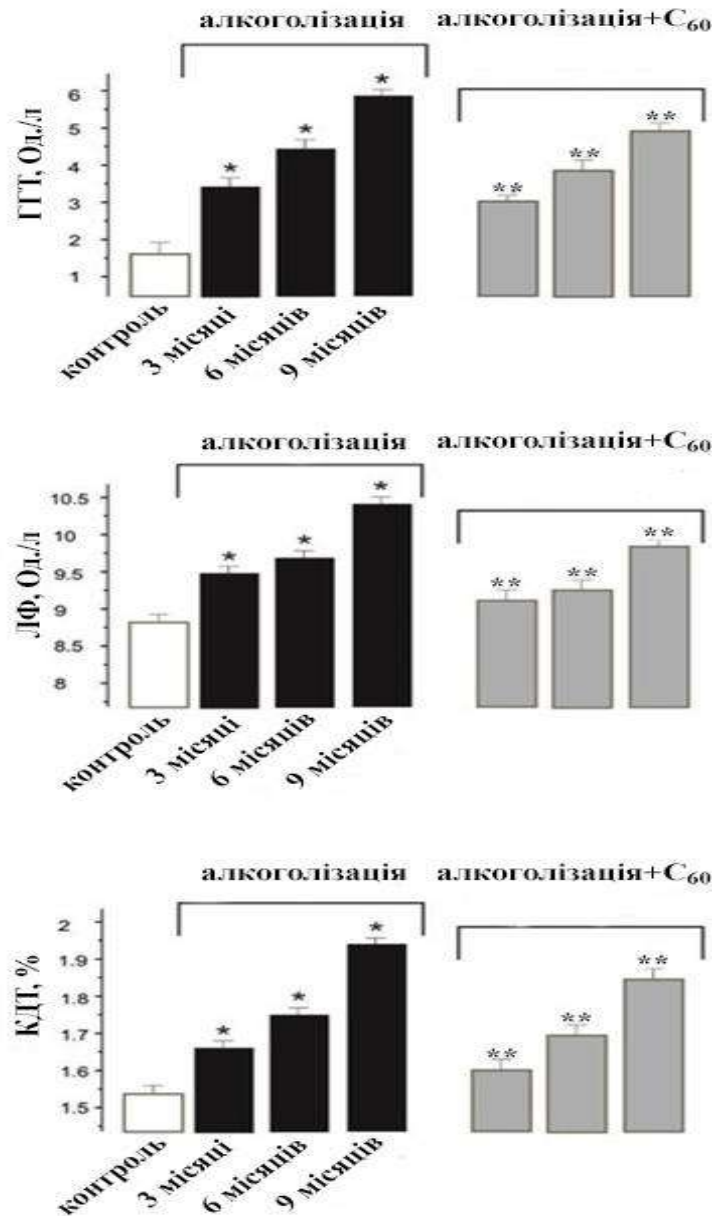


Рис. 13. Активності ферментів ГГТ і ЛФ та рівень КДТ у крові піддослідних тварин: алкоголізація — щури, які отримували алкоголь; алкоголізація + C₆₀ — щури, які отримували суміш етанолу і C₆₀ВРФ (1 мг/кг); 3, 6 і 9 місяців — алкоголізація упродовж 3, 6 і 9 місяців, відповідно. * $p < 0,05$ відносно контролю; ** $p < 0,05$ відносно групи алкоголізація.

Рівень КДТ є важливим маркером хронічної алкогольної інтоксикації. Перевагою його використання перед іншими полягає в тому, що він є найбільш специфічним до виявлення уражень печінки алкогольної етіології. Як бачимо, рівень КДТ

підвищився із $1,54 \pm 0,10\%$ у контролі до $1,65 \pm 0,1\%$, $1,75 \pm 0,1\%$ і $1,94 \pm 0,1\%$ за алкоголізації тварин тривалістю 3, 6 і 9 місяців, відповідно (рис. 13). Застосування C_{60} ВРФ знизило рівень цього показника в середньому на $10 \pm 1\%$ упродовж експеримента порівняно з групою алкоголізації.

Важливо зазначити, що застосування C_{60} ВРФ за оптимальної дози 1 мг/кг у профілактичній (за 1 год до вживання алкоголю) і терапевтичній (через 1 год після вживання алкоголю) схемах були менш ефективні щодо нормалізації патологічних станів печінки щурів на тлі їх хронічної алкогольної інтоксикації порівняно з дією C_{60} ВРФ за терапевтичної схеми (вживання разом з алкоголем).

У щурів контрольної групи гістопатологічних особливостей не спостерігали. Була присутня лише невелика кількість пікнотичних ядер у гепатоцитах (рис. 14А).

У печінці алкоголізованих щурів упродовж 9 місяців (найдовший термін експеримента) спостерігали сильну гідропічну дистрофію гепатоцитів зі зростанням їх розміру внаслідок набряку або збільшення функціонального навантаження на них (табл. 2; рис. 14Б). Деякі ядра клітин пікнотичні, інші збільшені та яскраві. Також у семи тварин спостерігали дрібнокраплинну ліпідну дистрофію, а у п'яти тварин — сильну крупнокраплинну ліпідну дистрофію та балонну дистрофію гепатоцитів (рис. 14Б). Некротичні гепатоцити виявлені у великій кількості тварин (10 з 12 щурів). Деякі кровоносні судини розширені, навколо них спостерігається інфільтрація лейкоцитів. Фіксується розростання сполучної тканини.

У печінці алкоголізованих щурів, які отримували C_{60} ВРФ упродовж 9 місяців, спостерігали помірно виражену гідропічну та слабо виражену дрібнокраплинну ліпідну дистрофію гепатоцитів, балонна дистрофія відсутня (рис. 14В). Гепатоцити з пікнотичними ядрами та некротичні гепатоцити присутні, але у значно меншій кількості, ніж в алкоголізованих тварин, які не отримували C_{60} ВРФ (табл. 2). Іноді спостерігали розширені кровоносні судини з лейкоцитарними інфільтратами навколо. Має місце слабе розростання сполучної тканини (рис. 14В). Отже, гістологічні зміни у печінці щурів, які отримували водорозчинні C_{60} фулерени разом з алкоголем, є значно меншими, що свідчить про їх гепатопротекторну дію.

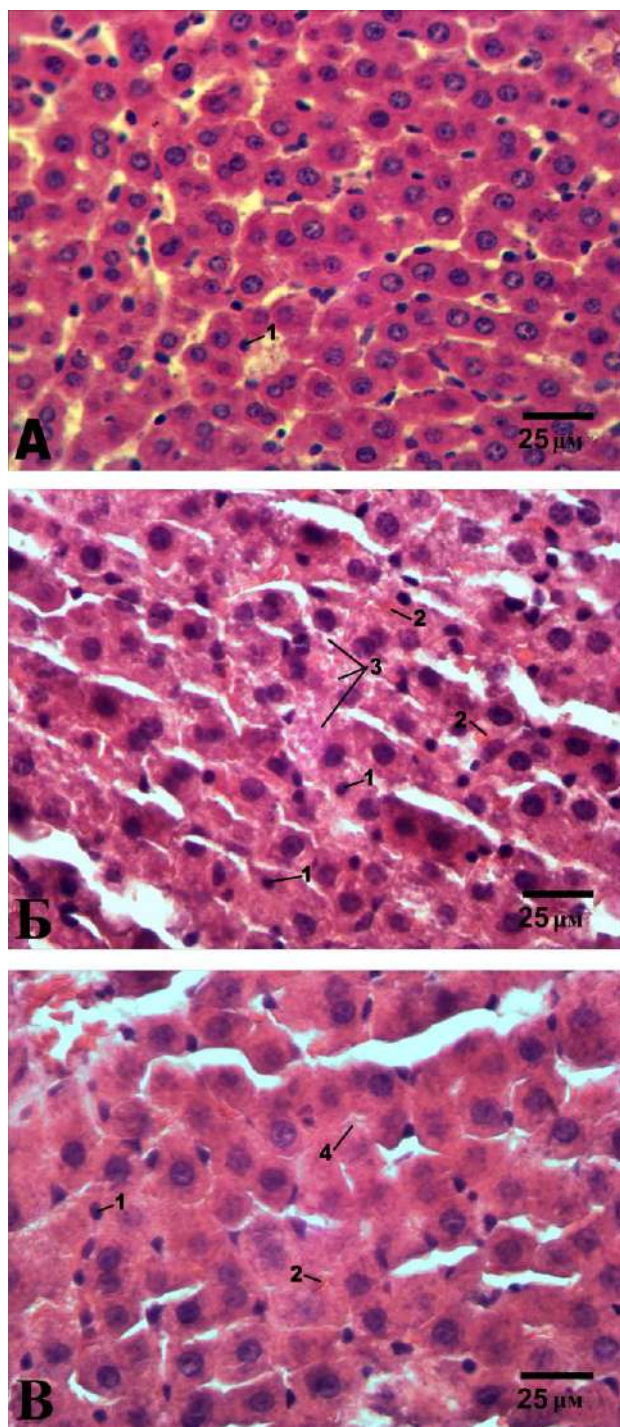


Рис. 14. Гістологічні зображення печінки піддослідних тварин: А — контрольна група; Б — група алкоголізація — щури, які отримували алкоголь упродовж 9 місяців; В - група алкоголізація + С₆₀ — щури, які отримували суміш етанолу і С₆₀ВРФ (1 мг/кг) упродовж 9 місяців; 1 — пікнотичні ядра; 2 — тільця Меллорі; 3 — ліпідна дистрофія з дрібними краплями та балонна дистрофія; 4 — гідропічна дистрофія. Фарбування гематоксиліном та еозином. Масштаб: 25 μм.

Таблиця 2. Гістологічні особливості печінки піддослідних щурів: алкоголізація — щури, які отримували алкоголь упродовж 9 місяців; алкоголізація + C_{60} — щури, які отримували суміш етанолу і C_{60} ВРФ (1 мг/кг) упродовж 9 місяців. $*p < 0,05$ відносно контролю; $**p < 0,05$ відносно групи алкоголізація. Інтенсивність проявів: «—» — не виявлено; «+» — слабка; «++» — помірна; «+++» — виражена.

Показник Групи	Контроль	Алкоголізація	Алкоголізація + C_{60}
Об'єм ядра гепатоцита, мкм ³	138 ± 8	$248 \pm 11^*$	$284 \pm 19^{**}$
Площа поперечного перерізу гепатоцита, мкм ²	205 ± 11	$273 \pm 20^*$	$244 \pm 11^{**}$
Пікнотичні ядра у гепатоцитах	+	++	+
Тільця Меллорі	—	+++	++
Ліпідна дистрофія гепатоцитів	—	+++	+
Гідропічна дистрофія гепатоцитів	—	+++	++
Балонна дистрофія гепатоцитів	—	++	—
Некротичні гепатоцити	—	++	+
Розширення кровоносних судин	—	++	+
Інфільтрація запальними клітинами	—	++	+
Розростання сполучної тканини (фіброз)	—	+	+

Отже, можна стверджувати, що запропонована терапевтична схема введення C_{60} ВРФ (1 мг/кг; разом з алкоголем) зменшує тяжкість перебігу патологічних

процесів у печінці щурів на тлі їх хронічної алкогольної інтоксикації, зокрема у всіх проведених тестах спостерігається позитивна динаміка біохімічних показників крові (в середньому на $15 \pm 2\%$), як маркерів ураження печінки, що підтверджується гістологічними даними.

РОЗДІЛ 4. Механізми дії водорозчинних C₆₀ фулеренів за хронічної алкогольної міопатії.

Відомо, що розвиток хронічної алкогольної міопатії пов'язаний зі зміною окисно-відновного стану та дисбалансом ендogenous антиоксидантів в організмі. Ці порушення викликають зниження вмісту АТФ у скелетних м'язах і зменшення окисно-відновного співвідношення НАД⁺/НАДН, що супроводжується збільшенням витоку електронів, головним чином із комплексу III (убіхінону), і сприяє утворенню мітохондріальних АФК та зворотному транспорту електронів. У сукупності ці процеси індукують мітохондріальні дисфункції скелетних м'язів, пригнічення синтеза білків та посилення аутофагії. Алкоголь посилює активність цитохрому P450, що також сприяє утворенню АФК. Нарешті, метаболізм етанолу в печінці призводить до утворення високореактивних сполук, а їх подальший метаболізм також пов'язаний з продукцією АФК.

Ефективна інактивація АФК наночастинками C₆₀ фулеренів у модельних системах *in vivo* відкриває реальні перспективи їх широкого застосування у біомедицині. Показано, що C₆₀ фулерен виявляє більш потужні антиоксидантні властивості порівняно з природними антиоксидантами — вітамінами С і Е, а також екзогенними антиоксидантами - N-ацетилцистеїн і β-аланін, які часто застосовують у спортивній медицині.

Слід зазначити, що мітохондрії є основними органелами, відповідальними за генерацію АФК у пошкоджених скелетних м'язах. Відтак, C₆₀ фулерени з мітохондріальним таргетуванням можуть виступати ефективними антиоксидантами у терапії м'язових патологій, опосередкованих окисним стресом.

Завданням цього етапу роботи було з'ясувати можливі механізми дії водорозчинних наночастинок C₆₀ фулеренів на *in vivo* моделі хронічної алкоголізації шляхом аналізу біохімічних показників крові піддослідних тварин та комп'ютерного моделювання.

Щоб перевірити антиоксидантну дію C₆₀ фулеренів, пов'язану з модифікацією АФК-залежних механізмів та їх впливом на активність ендogenous антиоксидантів, ми проаналізували показники про- та антиоксидантного балансу, а саме активності

СОД, каталази і ГП, концентрації відновленого глутатіона і H_2O_2 у крові щурів за тривалої алкогольної інтоксикації.

СОД є найпотужнішим природним антиоксидантом і ферментом першої лінії антиоксидантного захисту організму. Як бачимо з рис. 15, активність СОД збільшилася з $1,7 \pm 0,1$ Од./мл у контролі до $3,8 \pm 0,3$, $4,8 \pm 0,4$ і $5,9 \pm 0,5$ Од./мл за алкоголізації тварин упродовж 3, 6 і 9 місяців, відповідно. Застосування $\text{C}_{60}\text{ВРФ}$ (1 мг/кг; разом з алкоголем) знизило ці показники на $37 \pm 3\%$, $38 \pm 3\%$ та $39 \pm 3\%$ за алкоголізації тварин упродовж 3, 6 і 9 місяців, відповідно, порівняно з групою алкоголізація.

Каталаза запобігає утворенню високореактивних гідроксильних радикалів. Як бачимо з рис. 15, активність каталази зросла з $1,3 \pm 0,1$ мМ/хв до $2,2 \pm 0,1$, $2,5 \pm 0,1$ і $3,5 \pm 0,2$ мМ/хв за алкоголізації тварин упродовж 3, 6 і 9 місяців, відповідно. Застосування $\text{C}_{60}\text{ВРФ}$ знизило ці показники на $33 \pm 3\%$, $34 \pm 3\%$ і $37 \pm 3\%$ за алкоголізації тварин упродовж 3, 6 і 9 місяців, відповідно, порівняно з групою алкоголізація.

За розвитку хронічної алкогольної міопатії спостерігали значне підвищення концентрації відновленого глутатіона: вона зросла з $2,0 \pm 0,1$ μM у контролі до $3,2 \pm 0,2$, $3,9 \pm 0,2$ і $5,5 \pm 0,3$ μM за алкоголізації тварин упродовж 3, 6 і 9 місяців, відповідно. Застосування $\text{C}_{60}\text{ВРФ}$ знизило ці показники на $22 \pm 1\%$, $23 \pm 1\%$ і $31 \pm 3\%$ за алкоголізації тварин упродовж 3, 6 і 9 місяців, відповідно, порівняно з групою алкоголізація (рис. 15). Крім того, за дії $\text{C}_{60}\text{ВРФ}$ спостерігали також зниження активності ГП на $20 \pm 2\%$, $22 \pm 2\%$ і $26 \pm 2\%$ за алкоголізації тварин упродовж 3, 6 і 9 місяців, відповідно, порівняно з групою алкоголізація. Ці результати підтверджують здатність C_{60} фулеренів впливати на процеси синтезу і метаболізму глутатіона у м'язовій тканині за патологічних станів різного генезу.

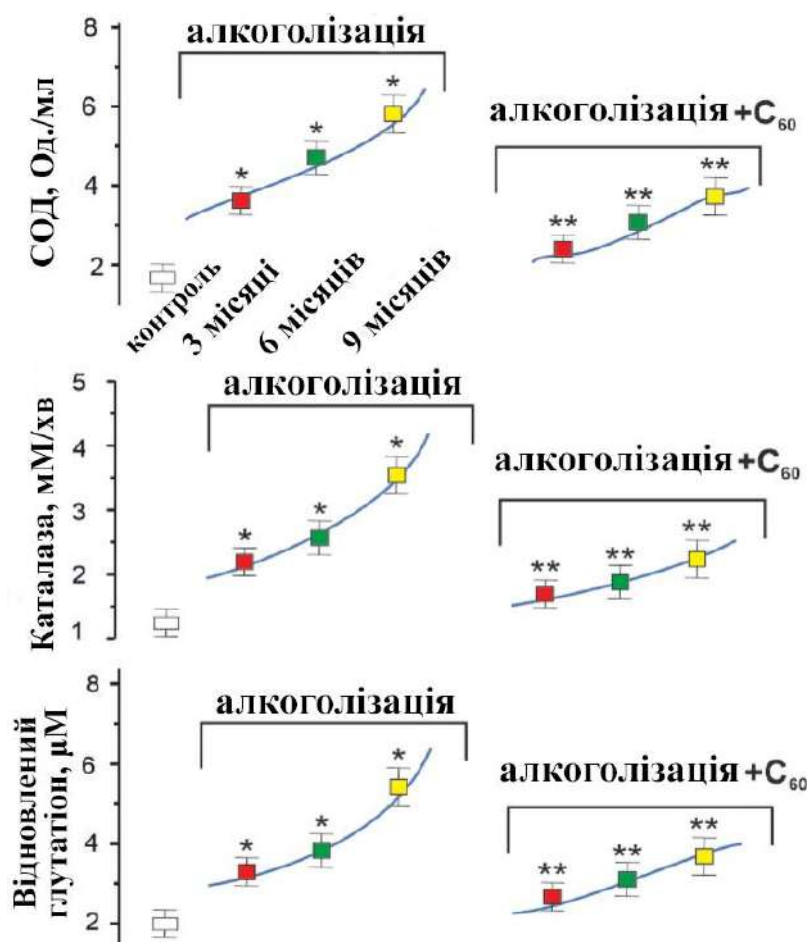


Рис. 15. Показники про- та антиоксидантного балансу (СОД, каталаза і відновлений глутатіон) у крові піддослідних тварин: алкоголізація — щури, які отримували алкоголь; алкоголізація + C₆₀ — щури, які отримували суміш етанолу і C₆₀ВРФ (1 мг/кг); 3, 6 і 9 місяців — алкоголізація упродовж 3, 6 і 9 місяців, відповідно. * $p < 0,05$ відносно контролю; ** $p < 0,05$ відносно групи алкоголізація.

Нарешті, застосування C₆₀ВРФ зменшило вміст H₂O₂ на $21 \pm 2\%$, $24 \pm 2\%$ і $29 \pm 2\%$ за алкоголізації тварин упродовж 3, 6 і 9 місяців, відповідно, порівняно з групою алкоголізація. Цей результат підтверджує той факт, що C₆₀ фулерени здатні проникати через мембрани клітин без ознак їх пошкодження.

Відомо, що саме активності СОД і ГП та концентрації ферментів, пов'язаних з відновленим глутатіоном, є мішенями регуляторного білка захисту клітин від окисного стресу - Nrf2. Як бачимо, водорозчинні наночастинки C₆₀ фулерена нормалізують рівень відновленого глутатіона та активність білків, пов'язаних з ним. Це свідчить про те, що поряд з іншими потенційними механізмами, антиоксидантний шлях

Nrf2/ARE може бути задіяний у регуляції гомеостазу глутатіона. Нарешті, можна припустити, що C₆₀ фулерени, як протизапальні агенти, здатні пригнічувати активацію транскрипційного фактора – регулятора запальних процесів у клітинах - NF-κB, зменшуючи таким чином продукцію прозапальних цитокінів, що підтверджується іншими авторами.

Проведені біохімічні дослідження виявили значне підвищення маркерів крові експериментальних тварин, пов'язаних з окисним стресом, на тлі розвитку хронічної алкогольної міопатії. Це свідчить, що м'язова система виконує роботу, яка є занадто інтенсивною для її фізіологічного рівня. За алкогольної міопатії АФК утворюються головним чином у дисфункціональних мітохондріях, що викликає руйнування клітинних мембран та функціональні порушення їх ферментних систем. Це може призвести до тимчасового порушення балансу у часі встановлення гладкого тетануса м'яза, що, у свою чергу, збільшує час, необхідний йому для виконання силової вправи. З іншого боку, відомо, що понад 90% спожитого алкоголю метаболізується в організмі окисними та неокисними шляхами, утворюючи такі хімічно активні сполуки, як ацетальдегід, ацетат, етиловий ефір жирної кислоти тощо. Ці сполуки також генерують АФК, які спричиняють підвищення окисного стресу та ПОЛ, порушуючи тим самим структурну цілісність клітин і функції тканин загалом. Покращення біохімічних показників (в середньому на $30 \pm 3\%$ порівняно з групою алкоголізація), досягнуте завдяки застосуванню екзогенних антиоксидантів C₆₀ фулеренів з мітохондріальним таргетуванням, демонструє їх здатність порушувати цикл окисного стресу та мітохондріальної дисфункції.

Підсумовуючи, можна запропонувати такі механізми дії C₆₀ фулеренів: (1) безпосередньо, ефективно інактивуючи АФК, та (2) опосередковано, впливаючи, наприклад, на Nrf2/ARE (стимулювання) і NF-κB (пригнічення) сигнальні шляхи, які беруть участь у регуляції антиоксидантних ферментів, C₆₀ фулерени нормалізують функціональний стан м'язової системи за розвитку хронічної алкогольної міопатії.

Результати МД моделювання показали, що поодинокі C₆₀ фулерени та їх нанокластери з 2, 3 і 4 молекул є нестабільними у середовищі «вода + етанол». Стабільними виявилися такі нанокластери (рис. 16, А-Г): 5 молекул C₆₀ (5C₆₀) розташовані у

вершинах трикутної біпіраміди, максимальна діагональ якої становить 2,2 нм; $10C_{60}$ і $13C_{60}$ є майже сферичними з діаметрами 2,5 і 2,9 нм, відповідно; $20C_{60}$ має еліпсоїдальну форму з найдовшою головною віссю 3,4 нм і двома іншими, приблизно рівними 2,5 нм. Мінімальна відстань між сусідніми C_{60} фулеренами у нанокластерах, які безпосередньо взаємодіють між собою, становить 0,27 нм, а максимальна – 0,32 нм.

Було розраховано кількість контактів між нанокластерами C_{60} і молекулами етанолу і води (табл. 3). Як бачимо, в середньому кількість контактів між нанокластерами C_{60} і водою лише на 23% більша, ніж з етанолом, що свідчить про здатність одержаних нанокластерів адсорбувати на своїй поверхні як молекули етанолу, так і молекули води. Найменша відстань між поверхнею нанокластерів C_{60} і молекулами етанолу становить 0,24 нм. Більшість ОН-груп етанолу орієнтуються на молекули води, прагнучи утворити з ними водневі зв'язки (рис. 16Д). Мережа цих водневих зв'язків стає щільнішою зі збільшенням розміру нанокластера. Загалом, кількість адсорбованих молекул етанолу напряму залежить від розміру нанокластерів C_{60} (рис. 16; табл. 3).

Підсумовуючи, можна запропонувати такий механізм дії C_{60} фулеренів. Етанол розповсюджується з кровотоку по всіх тканинах і рідинах організму. На концентрацію етанолу в тканинах впливає відносний вміст води. Подібно до води, етанол може проникати через клітинні мембрани. Важливо, що у крові відсутні білки, які зв'язуються з етанолом. З іншого боку, відомо, C_{60} фулерени та їх нанокластери здатні ефективно адсорбувати на своїй поверхні різні ароматичні молекули у водному середовищі та проникати всередину клітин. Відтак, високостабільні нанокластери C_{60} фулеренів (розміром 2,2-3,4 нм і більше) здатні ефективно зв'язувати молекули етанолу в організмі, виключаючи їх з метаболічного циклу, і, таким чином, зменшувати негативний вплив алкоголю на функціонування м'язової системи.

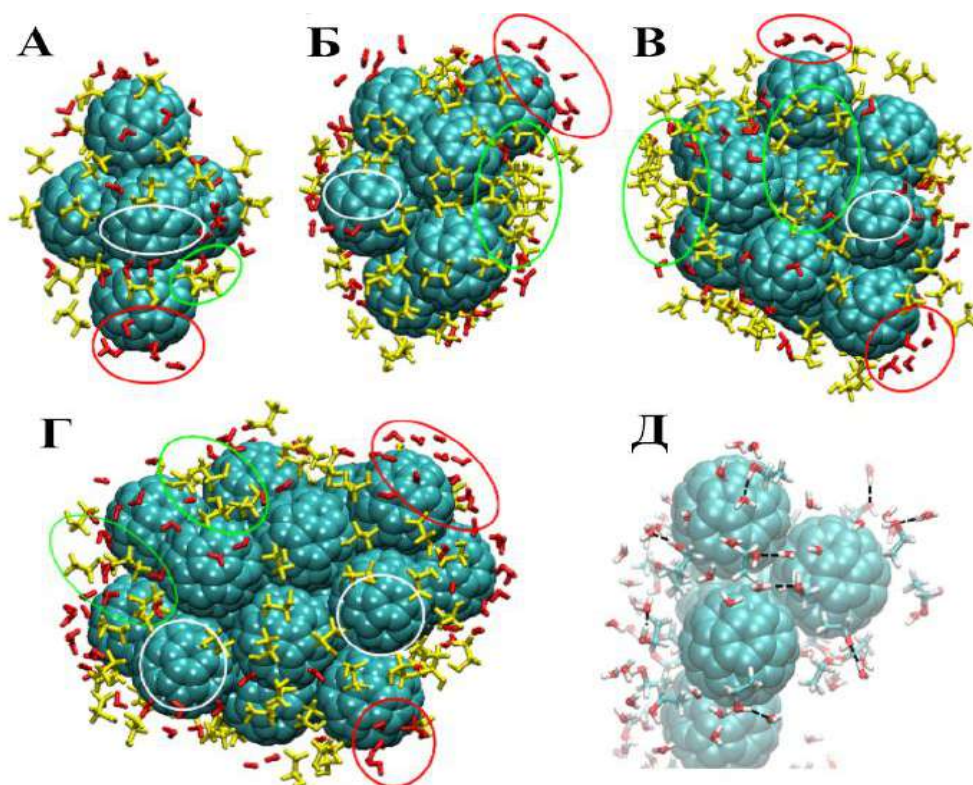


Рис. 16. Розраховані стабільні нанокластери $5C_{60}$ (А), $10C_{60}$ (Б), $13C_{60}$ (В) і $20C_{60}$ (Г). Молекули води позначені червоним кольором, етанол – жовтим. Кола позначають: білим – «сухі» ділянки, зеленим – порожнини між C_{60} фулеренами, червоним – опуклі поверхні. Фрагмент нанокластера $5C_{60}$ з першою оболонкою сольватації етанолу, де молекули зайняті водою в межах 0,24 нм (Д): чорні пунктирні лінії показують водневі зв'язки між ОН-групами етанолу та водою; атоми кисню позначені червоним кольором, атоми водню – білим, а атоми вуглецю – блакитним.

Таблиця 3. Розрахована середня кількість контактів між молекулами етанолу або води і C_{60} фулеренами залежно від їх кількості у нанокластері.

Кількість C_{60} фулеренів у нанокластері	Середня кількість контактів етанол – C_{60}	Середня кількість контактів вода – C_{60}
5	25 ± 4	29 ± 6
10	44 ± 5	67 ± 9
13	55 ± 6	74 ± 11
20	75 ± 6	93 ± 11

УЗАГАЛЬНЕННЯ

Представлені результати досліджень біомеханічних параметрів скоротливої активності скелетних м'язів, біохімічних показників крові та м'язової тканини, гістології м'язів і печінки щурів за тривалої алкогольної інтоксикації та дії водорозчинних C₆₀ фулеренів залежно від дози, схеми і перорального застосування.

Показано, що тривала алкоголізація тварин (упродовж 3, 6 і 9 місяців) викликає зниження силової відповіді м'язів порівняно з контролем. Так, наприклад, має місце зменшення значень імпульса сили *musculus gastrocnemius*, його мінімальної та максимальної сили скорочення на $74 \pm 4\%$, $91 \pm 5\%$ та $64 \pm 4\%$, відповідно, що вказує на значну м'язову дисфункцію за розвитку алкогольної міопатії упродовж 9 місяців. Водночас, застосування C₆₀ВРФ (щоденна пероральна доза 1 мг/кг разом з алкоголем) збільшило ці параметри на $26 \pm 2\%$, $33 \pm 3\%$ та $23 \pm 2\%$, відповідно, порівняно з групою алкоголізація.

Важливо зазначити, що за тривалої алкоголізації тварин зростає величина флукуаційних коливань сили скорочення *musculus gastrocnemius* на фазі утримання максимальної силової відповіді (так званий «тремор»): через 9 місяців вона становила $0,13 \pm 0,01$ мН (майже втричі перевищила контроль). У тварин, які отримували суміш алкоголю і C₆₀ВРФ, спостерігали значне зниження цього параметра, а саме на $66 \pm 3\%$ порівняно з групою алкоголізація.

Зафіксовано збільшення часових характеристик силової відповіді м'язів за тривалої алкоголізації тварин порівняно з контролем. Так, наприклад, значення часу початку силової відповіді *musculus gastrocnemius*, часу зниження сили його скорочення до нуля після припинення стимуляції, часу досягнення силою скорочення максимального рівня та часу встановлення гладкого тетануса зросли в середньому на $79 \pm 4\%$, $145 \pm 8\%$, $50 \pm 3\%$ та $15 \pm 1\%$, відповідно, що вказує на збільшення жорсткості м'яза, підвищення його втоми та порушення в активації сили скорочення окремими руховими одиницями за розвитку алкогольної міопатії упродовж 9 місяців. Застосування C₆₀ВРФ (щоденна пероральна доза 1 мг/кг разом з алкоголем) зменшило ці параметри на $20 \pm 2\%$, $41 \pm 3\%$ і $32 \pm 2\%$, відповідно, порівняно з групою алкоголізація, а час

встановлення гладкого тетануса *musculus gastrocnemius* майже наблизився до контролю.

Для підтвердження одержаних біомеханічних результатів було проаналізовано біохімічні показники крові, зокрема концентрації креатиніну, КФК, ЛДГ і ЛА, та про- та антиоксидантного балансу (концентрації H_2O_2 та відновленого глутатіону, активності СОД, каталази і ГП) у тканинах *musculus gastrocnemius*. Усі вони мають виражену тенденцію до зростання зі збільшенням тривалості алкоголізації тварин, що свідчить про значні пошкодження міоцитів. У тварин, які вживали суміш алкоголю і C_{60} ВРФ, ці показники виявилися значно нижчими порівняно з тваринами, які отримували лише алкоголь. Так, концентрація креатиніну знизилася на $37 \pm 2\%$, КФК - на $28 \pm 2\%$, ЛДГ - на $41 \pm 2\%$, ЛА - на $38 \pm 2\%$, H_2O_2 - на $51 \pm 2\%$ і відновленого глутатіону - на $42 \pm 2\%$, а активність СОД - на $44 \pm 2\%$, каталази - на $48 \pm 2\%$ і ГП - на $39 \pm 2\%$ за алкоголізації тварин упродовж 9 місяців порівняно з групою алкоголізації.

Зазначимо також, що одержані результати для *musculus soleus* (повільний м'яз) корелюють з динамікою вищеописаних біомеханічних і біохімічних даних для *musculus gastrocnemius* (швидкий м'яз) піддослідних тварин.

Гістологічний аналіз продемонстрував, що у щурів, які вживали алкоголь упродовж 9 місяців, більшість м'язових волокон тонші, ніж у контрольної групи, а середній діаметр волокон зменшується до 24,4 мкм. Є звивисті волокна. Деякі волокна мають нерівномірну товщину по всій довжині, а деякі - вакуолізовані, що свідчить про деструктивні процеси в них. Сполучна тканина між м'язовими волокнами збільшена, внаслідок чого площа, зайнята сполучною тканиною, зростає до 0,21 мкм² на 1 мкм² площі поперечного перерізу м'яза. Відтак, спостерігається склероз і фіброз. В алкоголізованих щурів, які отримували C_{60} ВРФ (щоденна пероральна доза 1 мг/кг разом з алкоголем), товщина м'язових волокон і площа, зайнята сполучною тканиною, незначно зменшилася і збільшилася, відповідно, порівняно з контрольною групою. Склероз і фіброз були менш вираженими.

Окремо була проаналізована тривала дія алкоголю на розвиток патологічних процесів у печінці щурів шляхом оцінки основних біохімічних показників крові, як маркерів дисфункції печінки, та гістологічних і морфометричних особливостей її

пошкодження. Так, наприклад, мало місце збільшення активностей АЛТ, АСТ, ГГТ і ЛФ та рівня КДТ на $58 \pm 3\%$, $15 \pm 1\%$, $73 \pm 4\%$ і $15 \pm 1\%$ та $11 \pm 1\%$, відповідно, порівняно з контролем, що є наслідком прогресуючого руйнування гепатоцитів. Застосування суміші алкоголю та C_{60} ВРФ знизило ці показники в середньому на $15 \pm 2\%$ упродовж експеримента порівняно з групою алкоголізації.

У печінці алкоголізованих щурів упродовж 9 місяців спостерігали сильну гідропічну дистрофію гепатоцитів зі зростанням розміру клітин. У деяких тварин спостерігали дрібнокраплинну/крупнокраплинну ліпідну дистрофію, а у інших - балонну дистрофію гепатоцитів. У більшості піддослідних тварин виявлено некротичні гепатоцити. Деякі кровоносні судини розширені, навколо них спостерігається інфільтрація лейкоцитів. Фіксується розростання сполучної тканини. Водночас, у печінці алкоголізованих щурів, які отримували C_{60} ВРФ (щоденна пероральна доза 1 мг/кг разом з алкоголем) упродовж 9 місяців, спостерігали помірно виражену гідропічну та слабо виражену дрібнокраплинну ліпідну дистрофію гепатоцитів, балонна дистрофія була відсутня, що свідчить про гепатопротекторну дію водорозчинних C_{60} фулеренів.

Підсумовуючи, можна стверджувати: одержані вище біомеханічні, біохімічні та гістологічні результати свідчать про значну дисфункцію скелетних м'язів і печінки за тривалої алкогольної інтоксикації організму, які насамперед проявляються у зниженні максимальної сили скорочення м'яза, порушенні динаміки розвитку тетануса, підвищенні м'язової втоми та появі «тремора», в активації процесів окисного стресу, атрофії м'язових волокон, порушенні структури міофібрил та деструкції гепатоцитів. Водночас, пероральне застосування водорозчинних C_{60} фулеренів за оптимальної дози 1 мг/кг та найбільш ефективної терапевтичної схеми (щоденно разом з алкоголем упродовж експеримента) здатне суттєво покращити скоротливу активність скелетних м'язів, зокрема зменшити амплітуду флуктуаційних коливань їх сили скорочення («тремор») та збільшити час до настання втоми, відновити біохімічні показники крові та м'язової тканини, запобігти розвитку тяжких патологічних станів печінки, викликаних алкогольною інтоксикацією.

Нарешті, для перевірки антиоксидантної дії C_{60} фулеренів, пов'язаної з модифікацією АФК-залежних механізмів та їх впливом на активність ендогенних

антиоксидантів, було проаналізовано показники про- та антиоксидантного балансу, а саме активності СОД, каталази і ГП, концентрації відновленого глутатіона і H_2O_2 у крові щурів за тривалої алкогольної інтоксикації. Так, наприклад, активності СОД і каталази зросли майже в 3,5 і 2,7 рази, відповідно, а концентрація відновленого глутатіона – у 2,8 рази порівняно з контролем за розвитку алкогольної міопатії упродовж 9 місяців. Застосування C_{60} ВРФ (щоденна пероральна доза 1 мг/кг разом з алкоголем) знизило ці показники на $39 \pm 3\%$, $37 \pm 3\%$ і $31 \pm 3\%$, відповідно, порівняно з групою алкоголізація.

З іншого боку, в рамках комп'ютерного експеримента було встановлено, що C_{60} фулерени утворюють стабільні нанокластери, які містять 5 і більше молекул, розміром 2,2 нм і більше у водному середовищі. Показано, що такі нанокластери C_{60} фулеренів здатні адсорбувати на своїй поверхні молекули етанолу, причому тим більшу кількість, чим більший їхній розмір.

Враховуючи ці результати, можна запропонувати такі можливі механізми дії C_{60} фулеренів: (1) безпосередньо, ефективно інактивуючи АФК, та (2) опосередковано, впливаючи на сигнальні шляхи, які беруть участь у регуляції антиоксидантних ферментів, C_{60} фулерени здатні нормалізувати функціональний стан м'язової системи за розвитку хронічної алкогольної міопатії; (3) стабільні нанокластери C_{60} фулеренів, потрапляючи в організм, здатні ефективно зв'язувати молекули етанолу, виключаючи їх з метаболічного циклу, і, таким чином, зменшувати негативний вплив алкоголю на організм загалом і скоротливу активність скелетних м'язів, зокрема.

ВИСНОВКИ

У дисертаційній роботі детально проаналізовано основні біомеханічні параметри скоротливої активності скелетних м'язів (швидкі та повільні), біохімічні показників крові та м'язової тканини, гістології м'язів та печінки щурів за тривалої алкогольної інтоксикації та дії водорозчинних C_{60} фулеренів залежно від дози, схеми (до вживання алкоголю, вживання разом з акоголем і після вживання алкоголю) та перорального застосування. Одержані *in vivo* результати вказують на можливість використання цих вуглецевих наночастинок для корекції патологічних станів скелетних м'язів та супутніх метаболічних порушень, викликаних алкогольною інтоксикацією, що потребує подальших клінічних випробувань.

Вперше показано, що тривале вживання алкоголю (3, 6 і 9 місяців) викликає:

1. значні зміни у скоротливій активності скелетних м'язів щурів обох типів (повільний та швидкий) порівняно з контролем, які проявляються насамперед у зниженні максимальної і мінімальної сили скорочення, порушенні динаміки розвитку гладкого тетануса, підвищенні втоми та появі «тремора». Застосування водорозчинних C_{60} фулеренів у терапевтичній схемі введення (пероральна щоденна доза 1 мг/кг разом з алкоголем упродовж експеримента), яка виявилася найбільш ефективною, нормалізує час встановлення гладкого тетануса м'язів, покращує біомеханічні параметри їх силової відповіді в середньому на $35 \pm 3\%$, зокрема зменшуючи «тремор» в середньому на $60 \pm 6\%$ та подовжуючи час виникнення втоми у м'язах в середньому на $25 \pm 2\%$ порівняно з групою алкоголізація упродовж 9 місяців експеримента;

2. значне зростання досліджуваних біохімічних показників крові та м'язової тканини, як маркерів дисфункції м'язів, порівняно з контролем, що свідчить про активацію процесів окисного стресу та ПОЛ. Гістологічний аналіз скелетних м'язів виявив атрофію м'язових волокон, вакуолізацію саркоплазми та порушення структури міофібрил, що підтверджує розвиток хронічної алкогольної міопатії. Застосування водорозчинних C_{60} фулеренів у терапевтичній схемі введення (пероральна щоденна доза 1 мг/кг разом з алкоголем упродовж експеримента) відновлює біохімічні показники крові (концентрації креатиніну, КФК, ЛДГ і ЛА) та

м'язової тканини (концентрації H_2O_2 і відновленого глутатіона, активності СОД, каталази і ГП) в середньому на $30 \pm 3\%$ порівняно з групою алкоголізація упродовж 9 місяців експеримента. Дані гістологічного аналізу засвідчили, що товщина м'язових волокон і площа, зайнята сполучною тканиною, незначно зменшилася і збільшилася, відповідно, порівняно з контрольною групою. Склероз і фіброз були менш вираженими порівняно з групою алкоголізація упродовж 9 місяців експеримента;

3. значні порушення у функціонуванні печінки щурів порівняно з контролем, про що свідчить зростання біохімічних показників крові - ферментів-маркерів її ураження. У печінці алкоголізованих щурів упродовж 9 місяців спостерігали сильну гідропічну дистрофію гепатоцитів зі зростанням розміру клітин, дрібнокраплинну/крупнокраплинну ліпідну дистрофію та балонну дистрофію. Виявлено некротичні гепатоцити, фіксується розростання сполучної тканини. Застосування водорозчинних C_{60} фулеренів у терапевтичній схемі введення (пероральна щоденна доза 1 мг/кг разом з алкоголем упродовж експеримента) знижує активності АЛТ, АСТ, ГГТ і ЛФ та рівень КДТ в середньому на $15 \pm 2\%$ упродовж експеримента порівняно з групою алкоголізація. У печінці спостерігали помірно виражену гідропічну та слабо виражену дрібнокраплинну ліпідну дистрофію гепатоцитів, балонна дистрофія була відсутня, що свідчить про гепатопротекторну дію водорозчинних C_{60} фулеренів;

4. значне зростання показників про- та антиоксидантного балансу (активності СОД, каталази і ГП, концентрації відновленого глутатіона і H_2O_2) у крові щурів. Застосування водорозчинних C_{60} фулеренів у терапевтичній схемі введення (пероральна щоденна доза 1 мг/кг разом з алкоголем упродовж експеримента) знизило їх значення в середньому на $30 \pm 3\%$ за алкоголізації тварин упродовж 9 місяців порівняно з групою алкоголізація. Це вказує на те, що C_{60} фулерени, як антиоксиданти, з одного боку, здатні ефективно інактивувати АФК, індуковані алкогольною інтоксикацією організму, а з іншого, - активувати захисну дію антиоксидантної системи у відповідь на розвиток цієї патології. Нарешті, модельні розрахунки показали, що наночастинки C_{60} фулеренів здатні ефективно зв'язувати молекули етанолу, виключаючи їх з метаболічного циклу, і, таким чином, зменшувати негативний вплив алкоголю на функціонування скелетних м'язів.

СПИСОК ОПУБЛІКОВАНИХ ПРАЦЬ ЗА ТЕМОЮ ДИСЕРТАЦІЇ

Праці, в яких опубліковані основні наукові результати дисертації

1. **O. Motuziuk**, D. Nozdrenko, S. Prylutska, I. Vareniuk, K. Bogutska, S. Braniuk, O. Korotkyi, Yu. Prylutskyu, U. Ritter, J. Piosik. The effect of C₆₀ fullerene on the mechanokinetics of *muscle gastrocnemius* contraction in chronically alcoholized rats. *Heliyon*, 2023;9(8):e18745. **(Q1)** (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).
2. **O. Motuziuk**, D. Nozdrenko, S. Prylutska, K. Bogutska, I. Mishchenko, O. Abramchuk, S. Khrapaty, U. Ritter, Yu. Prylutskyu. C₆₀ fullerene reduces the level of fluctuations in the force response of *muscle gastrocnemius* in chronically alcoholized rats. *Applied Nanoscience*, 2023;13(10):7057-7067. **(Q2)** (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).
3. D. Nozdrenko, **O. Motuziuk**, S. Prylutska, T. Matvienko, K. Bogutska, D. Franskevych, N. Nurishchenko, O. Abramchuk, Yu. Prylutskyu. C₆₀ fullerene protective effect against the rat *muscle soleus* trauma. *Heliyon*, 2024;10(11):e32677. **(Q1)** (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).
4. **O. Motuziuk**, D. Nozdrenko, S. Prylutska, I. Vareniuk, V. Cherepanov, K. Bogutska, S. Rudenko, Yu. Prylutskyu, J. Piosik, U. Ritter. C₆₀ fullerene reduces the level of liver damage in chronic alcohol intoxication of rats. *Molecules*, 2024;29(13):2951. **(Q1)** (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).
5. Yu. Prylutskyu, D. Nozdrenko, O. Omelchuk, S. Prylutska, **O. Motuziuk**, V. Soroca, I. Vareniuk, V. Stetska, K. Bogutska, U. Ritter, J. Piosik. Effect of C₆₀ fullerene on

muscle injury-induced rhabdomyolysis and associated acute renal failure. *International Journal of Nanomedicine*, 2024;19:8043-8058. (**Q1**) (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

6. Yu. Prylutskyu, D. Nozdrenko, **O. Motuziuk**, S. Prylutska, K. Bogutska, O. Abramchuk, A. Morenko, D. Franskevych, P. Scharff, U. Ritter. C₆₀ fullerene reduces the development of post-traumatic dysfunction rat *muscle soleus*. *International Journal of Molecular Sciences*, 2024;25(22):12206. (**Q1**) (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

7. Yu. Prylutskyu, D. Nozdrenko, **O. Motuziuk**, S. Prylutska, N. Nurishchenko, D. Franskevych, V. Soroca, V. Cherepanov, I. Kalinin, O. Korzhyk, U. Ritter. C₆₀ fullerene improves the contractile activity of the injured rat *muscle gastrocnemius*. *Nanotechnology*, 2025;36(12):125101. (**Q2**) (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

8. Yu. Prylutskyu, D. Nozdrenko, **O. Motuziuk**, S. Prylutska, I. Vareniuk, N. Nurishchenko, D. Franskevych, V. Soroca, K. Bogutska, U. Ritter. C₆₀ fullerene promotes post-traumatic recovery of the rat *muscle gastrocnemius*. *Nanomedicine*, 2025;20(6):571-584. (**Q2**) (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

9. **O. Motuziuk**, D. Nozdrenko, S. Prylutska, Y. Osyp, K. Bogutska, O. Dmytrotsa, Yu. Prylutskyu, P. Scharff, U. Ritter. C₆₀ fullerenes diminish dysfunctions in rat *muscle soleus* with chronic alcohol intoxication. *Journal of Nanotechnology*, 2025;2025:Article ID 9916259. (**Q2**) (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

10. **O. Motuziuk**, D. Nozdrenko, S. Prylutska, N. Tverdokhlebo, M. Petrovsky, S. Vyzhva, Yu. Prylutskyu, P. Scharff, U. Ritter. The effect of C₆₀ fullerene on the *musculus gastrocnemius* contraction in chronically alcohol-exposed rats and the potential mechanism of its action. *Scientific Reports*, 2025;15:32651. **(Q1)** (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань та модельних розрахунків, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

Праці, які додатково відображають наукові результати дисертації

Статті:

1. O. Melnychuk, **O. Motuziuk**, S. Shvayko, S. Zay. Progressive fatigue dynamic development of isolated natively *musculus gastrocnemius medialis* in alcoholic rats. *Науковий вісник Східноєвропейського національного університету*. 2015;2(302):179–184. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).
2. О. Мельничук, **О. Мотузіук**, Т. Поручинська. Аналіз інтенсивності змін силової продуктивності *m. gastrocnemius* в алкоголізованих щурів із 6-годинною васкулярною ішемією задніх кінцівок. *Вісник Львівського університету. Серія біологічна*. 2015;69:27–40. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).
3. О.А. Мельничук, **О.П. Мотузіук**, С.Е. Швайко. Зміна параметрів тетанічного скорочення *m. soleus* в алкоголізованих щурів за умов унілатеральної васкулярної ішемії м'язів задніх кінцівок. *Фізіологічний журнал*. 2015;61(3):81–90. **(Scopus)**. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).
4. S. Zay, D. Zavodovskyi, D. Nozdrenko, K. Bogutska, **O. Motuziuk**, Yu. Sklyarov, Yu. Prylutskyu. Chronic alcoholization: effect on musculoskeletal system and remedial

action of C₆₀ fullerenes. *International Journal of Physiology and Pathophysiology*. 2016;7(3):273–284. (Участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

5. С. Зай, **О. Мотузок**, В. Білобров, Д. Улицька, О. Ноздренко, О. Абрамчук. Зміна швидкісно-силових параметрів скорочення *musculus soleus* щурів при хронічній алкоголізації. *Науковий вісник Східноєвропейського національного університету*. 2017;13(362):90–97. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

6. С. Зай, **О. Мотузок**, В. Білобров, Д. Улицька, О. Ноздренко, О. Абрамчук. Вплив ВРС₆₀ на швидкісно-силові параметри тетанічного скорочення *m. soleus* алкоголізованих щурів за умови експериментально-індукованої ішемії різної тривалості. *Вісник Київського національного університету імені Тараса Шевченка. Проблеми регуляції фізіологічних функцій*. 2017;2(23):59–64. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

7. С. Зай, Т. Матвієнко, В. Білобров, Д. Улицька, **О. Мотузок**. Динаміка скорочення *m. gastrocnemius medialis* щурів при хронічній алкоголізації. *Науковий вісник Східноєвропейського національного університету*. 2017;7(356):215–221. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

8. С.Ю. Зай, **О.П. Мотузок**, В.Ю. Білобров, Д.А. Вулицька, О.А. Ноздренко, О.М. Абрамчук. Зміни силової відповіді *musculus soleus* у алкоголізованих щурів з експериментально-індукованою ішемією тривалістю 1 та 2 години. *Вісник Харківського національного університету імені В.Н. Каразіна. Серія «Біологія»*. 2017;29:82–88. (Особисто дисертантом запропоновано ідею дослідження, участь у

проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

9. Yu.I. Prylutskyu, D.M. Nozdrenko, S.Yu. Zay, **O.P. Motuziuk**, K.I. Bogutska, A.V. Ilchenko. Influence of C₆₀ fullerenes on the mechanokinetic and biochemical parameters of *muscle soleus* contraction in chronically alcoholised rats with experimentally-induced ischemia. *Наносистеми, наноматеріали, нанотехнології*. 2018;16(3):583–596.

(Scopus). (Участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

10. **О. Мотузюк**, В.С. Пикалюк. Гістологічні особливості *m. gastrocnemius* щура за умов хронічної алкоголізації та ішемії різної тривалості. *Науковий вісник Східноєвропейського національного університету імені Лесі Українки. Серія: Біологічні науки*. 2019;3(387):137–147. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

11. **О. Мотузюк**, Д. Мельничук. Гістологічні особливості волокон литкового м'язу щурів при ішемії та використанні C₆₀ фулеренів в ролі антиоксидантів. *Науковий вісник Східноєвропейського національного університету імені Лесі Українки. Серія: Біологічні науки*. 2019;4(388):84–96. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

12. D.M. Nozdrenko, **O.P. Motuziuk**, K.I. Bogutska, V.L. Osetskyi, Yu.I. Prylutskyu. Dynamics of contraction of the *musculus soleus* of rats in chronic alcoholization and therapeutic action of water-soluble C₆₀ fullerenes. *Nanosistemi, Nanomateriali, Nanotehnologii*. 2019;17(2):399–408. **(Scopus)**. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

13. **O.P. Motuziuk**, D.M. Nozdrenko, K.I. Bogutska, O.V. Remeniak, Yu.I. Prylutskyu. Morphological changes in the ischemic muscle fibres of *musculus soleus* at chronic alcoholization of animals and application of an aqueous colloidal solution of C₆₀ fullerenes. *Nanosistemi, Nanomateriali, Nanotehnologii*. 2019;17(4):697–708. **(Scopus)**. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).
14. N. Wang, **O. Motuziuk**, I. Mischenko. The bibliometric analysis of the alcohol-induced muscle disease based on CiteSpace. *Науковий вісник Східноєвропейського національного університету імені Лесі Українки. Серія: Біологічні науки*. 2020;2(389):83–91. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).
15. N. Wang, **O. Motuziuk**, I. Mishchenko, Y. Zhou. Levels of relevant inflammatory factors and oxidative stress markers in alcoholic myopathy: a meta-analysis. *Chinese General Practice*. 2022;25(33):4123–4129. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).
16. **O. Motuziuk**, D. Nozdrenko, S. Prylutska, K. Bogutska, O. Korotkyi, Yu. Prylutskyu. Biochemical parameters of blood and tissue of the *muscle gastrocnemius* in chronically alcoholized rats under oral administration of C₆₀ fullerene aqueous solution. *Ukrainian Biochemical Journal*. 2023;95(2):58–67. **(Scopus)**. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).
17. **О.П. Мотузіук**, Д.М. Ноздренко, К.І. Богуцька, Н.Е. Нурищенко, В.Л. Осецький, Ю.І. Прилуцький. Біомеханічні параметри розвитку втомлювальних процесів у *muscle gastrocnemius* щурів за хронічної алкоголізації та застосування водорозчинних C₆₀ фулеренів. *Наносистеми, наноматеріали, нанотехнології*.

2023;21(3). **(Scopus)**. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

18. D. Nozdrenko, N. Wang, **O. Motuziuk**, S. Prylutska, O. Vygovska, K. Bogutska, O. Dmytrotsa, O. Lynchak, Y. Prylutskyu. The effect of C₆₀ fullerenes on the recovery of *muscle soleus* contraction dynamics in rats after chronic alcoholization. *Біологічні студії/ Studia Biologica*. 2024;18(1):43–56. **(Scopus)**. (Особисто дисертантом запропоновано ідею дослідження, участь у проведенні експериментальних вимірювань, аналіз та обговорення отриманих результатів, формулювання висновків та написання статті).

Тези наукових доповідей:

1. О.А. Мельничук, **О.П. Мотузіук**. Силові характеристики скорочення *m. gastrocnemius* у алкоголізованих щурів за умов унілатеральної васкулярної ішемії. *Актуальні питання патології за умов дії надзвичайних факторів на організм: матеріали IV наук.-практ. конф.* – Тернопіль, 2013. – С. 142–145.

2. О.А. Мельничук, Н.Г. Савіцька, **О.П. Мотузіук**. Вплив етилового спирту на швидкість досягнення максимальної сили *m. gastrocnemius* за умов васкулярної ішемії. *Біологічні дослідження – 2014: збірник V Всеукр. наук.-практ. конф.* – Житомир: ЖДУ ім. І. Франка, 2014. – С. 354–357.

3. О.А. Мельничук, **О.П. Мотузіук**. Зміна контрактильних характеристик функціонально-спеціалізованих скелетних м'язів у алкоголізованих щурів за умов васкулярної ішемії задніх кінцівок. *Сучасні проблеми викладання та наукових досліджень біології у ВНЗ України: матеріали I Всеукр. наук.-практ. конф.* – Дніпро: Вид-во ДНУ, 2014. – С. 58–61.

4. О.А. Melnychuk, S.Ye. Shvayko, N.H. Savitska, **O.P. Motuziuk**. The change of successive muscular contraction of frequency summation in ischemic *m. gastrocnemius medialis* in alcoholic rats. *Біологічні дослідження 2015: збірник наук. праць.* – Житомир: ПП «Рута», 2015. – С. 341–344.

5. О. Melnychuk, **О. Motuziuk**, S. Shvayko. Frequency summation efficiency of successive muscular contraction of *m. gastrocnemius medialis* (MGM) tetanus in alcoholic rats. *Матеріали VI з'їзду Українського біофізичного товариства* (27–30 травня 2015 р., Луцьк–Світязь, Україна). – Луцьк–Світязь, 2015. – С. 86.
6. О. Мельничук, **О. Мотузок**, С. Швайко. Вплив хронічної алкогольної інтоксикації на характер зміни силової продуктивності ішемізованого *m. gastrocnemius medialis* у лабораторних щурів. *Матеріали XI Міжнар. конф.* (20–24 квітня 2015 р., Львів). – Львів, 2015. – С. 477–478.
7. С.Ю. Зай, **О.П. Мотузок**, Ю.В. Лукницька. Особливості тетанічного скорочення ішемізованого *m. soleus* алкоголізованих щурів за дії водного розчину C_{60} фулеренів. *Біологія: від молекули до біосфери: тези доповідей XI Міжнар. конф.* (26 листопада – 2 грудня 2016 р., Харків). – Харків: ХНУ ім. В.Н. Каразіна, 2016. – С. 50–51.
8. І. Міщенко, **О. Мотузок**, А. Линник. Розвиток втоми скелетних м'язів за дії наночастинок C_{60} фулеренів. *XXI Міжнародний медичний конгрес.* – Тернопіль: Укрмедкнига, 2017. – С. 256.
9. І. Міщенко, **О. Мотузок**. Вплив C_{60} фулеренів на розвиток втоми скелетних м'язів. *Фізіологія – медицині, фармації та педагогіці: актуальні проблеми та сучасні досягнення.* – Харків, 2017. – С. 87–88.
10. І. Міщенко, **О. Мотузок**, А. Линник. Порівняльний аналіз антиоксидантних властивостей наночастинок C_{60} фулеренів і N-ацетилцистеїну в умовах розвитку м'язової втоми. *Здобутки клінічної та експериментальної медицини*, 14 червня 2017 р. – Тернопіль: ТДМУ, 2017. – С. 328–330.
11. О. Дубнік, **О. Мотузок**. Морфологічні зміни в скелетних м'язах за умов ішемії різної тривалості та алкогольної інтоксикації. *Молода наука Волині: пріоритети та перспективи досліджень: матеріали XI Міжнар. конф.* (16–17 травня 2017 р.). – Луцьк: СНУ ім. Лесі Українки, 2017. – С. 287–289.
12. О.М. Подпалова, **О.П. Мотузок**, О.Д. Ноздренко. Біомеханічні маркери рівня алкогольної міопатії при хронічній алкоголізації щурів. *Матеріали чергового VIII з'їзду Українського біофізичного товариства.* – Київ, 2019. – С. 52.

13. Д. Ноздренко, Ю. Прилуцький, **О. Мотузіук**. Терапевтичний вплив водного розчину C₆₀ фулеренів на скорочення *m. soleus* щурів за умов хронічної алкоголізації. *Матеріали чергового VIII з'їзду Українського біофізичного товариства*. – Київ, 2019. – С. 45.
14. **О.П. Мотузіук**, С.Ю. Зай, О.Д. Ноздренко. Різниця скоротливої активності *m. soleus* алкоголізованих щурів при різних ступенях ішемізації м'яза. *Матеріали чергового VIII з'їзду Українського біофізичного товариства*. – Київ, 2019. – С. 47.
15. **О.П. Мотузіук**, В.С. Пикалюк. Гістологічні особливості *m. gastrocnemius* щура за умов хронічної алкоголізації та ішемії різної тривалості. *Збірник тез VII Конгресу Наукового товариства анатомів, гістологів, ембріологів, топографоанатомів України*. – Одеса, 2019. – С. 68.
16. В.С. Пикалюк, **О.П. Мотузіук**, О.А. Мельничук. Гістологічні особливості *m. gastrocnemius* щура за умов хронічної алкоголізації та ішемії різної тривалості. *Прикладні аспекти морфології експериментальних і клінічних досліджень: матеріали наук.-практ. конф.* – Тернопіль, 2019. – С. 139–140.
17. **О. Motuziuk**, D. Nozdrenko, K. Bogutska, U. Ritter, Yu. Prylutsky. C₆₀ fullerene effect on the degree of tremor oscillations of muscle contraction in chronic alcoholic rats. International conference “NANO–2022”, 25–27 August 2022, Lviv. – Kyiv: LLC APF Polygraph Service, 2022. – P. 247.
18. **О. Motuziuk**, D. Nozdrenko, I. Vareniuk, K. Bogutska, Yu. Prylutsky. C₆₀ fullerene effect on the mechanokinetics of *muscle gastrocnemius* contraction in chronically alcoholized rats. International conference “NANO–2023”, 16–19 August 2023, Bukovel. – Kyiv: LLC APF Polygraph Service, 2023. – P. 292–293.
19. **О. Motuziuk**, D. Nozdrenko, I. Vareniuk, V. Cherepanov, U. Ritter, Yu. Prylutsky. C₆₀ fullerene reduces the level of liver damage in chronic alcohol intoxication of rats. International conference “NANO–2024”, 21–24 August 2024, Uzhgorod. – Kyiv: LLC APF Polygraph Service, 2024. – P. 307–308.
20. **О. Motuziuk**, D. Nozdrenko, N. Tverdokhle, U. Ritter, Y. Prylutsky. C₆₀ fullerene effect on the *musculus gastrocnemius* contraction in chronically alcohol-exposed rats and its mechanism of action. International Research and Practice

Conference “Nanotechnologies and Nanomaterials” (NANO–2025): Abstract Book /
Edited by Dr. Olena Fesenko. – Bukovel, August 20–23, 2025. – Kyiv: LLC APF
Polygraph Service, 2025. – P. 230-231.

ДОДАТКИ
ОСНОВНІ ПУБЛІКАЦІЇ ЗА ТЕМОЮ ДИСЕРТАЦІЇ



The effect of C₆₀ fullerene on the mechanokinetics of *muscle gastrocnemius* contraction in chronically alcoholized rats

Olexandr Motuziuk^{a,b}, Dmytro Nozdrenko^b, Svitlana Prylutska^c, Igor Vareniuk^b,
Kateryna Bogutska^b, Serhii Braniuk^a, Olexandr Korotkyi^b, Yuriy Prylutskyi^{b,**},
Uwe Ritter^d, Jacek Piosik^{e,*}

^a Faculty of Biology and Forestry, Lesya Ukrainka Volyn National University, Lutsk, 43025, Ukraine

^b Department of Biophysics and Medical Informatics, ESC "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv, Kyiv, 01601, Ukraine

^c Department of Physiology, Plant Biochemistry and Bioenergetics, Faculty of Plant Protection, Biotechnology and Ecology, National University of Life and Environmental Science of Ukraine, Kyiv, 03041, Ukraine

^d Institute of Chemistry and Biotechnology, Technical University of Ilmenau, Ilmenau, 98693, Germany

^e Intercollegiate Faculty of Biotechnology, University of Gdansk, 80-307, Gdańsk, Poland

ARTICLE INFO

Keywords:

C₆₀ fullerene

m. gastrocnemius

Biomechanical and biochemical parameters

Histological analysis

ABSTRACT

The C₆₀ fullerene effect (oral administration at a dose of 1 mg kg⁻¹) on the selected biomechanical parameters of *muscle gastrocnemius* contraction, biochemical indicators of blood and muscle tissue as well as histological changes in rat muscle tissue after chronic alcoholization for 3, 6 and 9 months was studied in detail. Water-soluble C₆₀ fullerenes were shown to reduce the pathological processes development in the muscle apparatus by an average of (35–40)%. In particular, they reduced the time occurrence of fatigue processes in muscle during the long-term development of alcoholic myopathy and inhibited oxidative processes in muscle, thereby preventing its degradation. These findings open up the possibility of using C₆₀ fullerenes as potent antioxidants for the correction of the pathological conditions of the muscle system arising from alcohol intoxication.

1. Introduction

Chronic alcoholic myopathy develops in (40–60)% of alcohol abusers and is accompanied by decreased performance, proximal paresis, and skeletal muscle atrophy. It remains unclear whether the duration or amount of alcohol consumption is important for the development of chronic alcoholic myopathy. The chronic course of this pathological process in skeletal muscle is also unknown. Studies in men have shown that chronic alcoholic myopathy develops after 5 years of alcohol abuse [1].

Ethanol is a toxic substance, it causes direct and indirect effects on the muscular system, which leads to alcoholic myopathy. The metabolism of alcohol is closely related to enzymes involved in oxidative stress and the generation of reactive oxygen species (ROS), which cause damage to cells and tissues [2]. Oxidative stress can be considered the consequence of ROS formation imbalance and the antioxidant defense system. Alcohol is mainly metabolized in the liver by alcohol dehydrogenase enzymes. At the same time, ethanol

* Corresponding author.

** Corresponding author.

E-mail addresses: prylut@ukr.net (Y. Prylutskyi), jacek.piosik@ug.edu.pl (J. Piosik).

oxidation, especially in patients with chronic alcoholism, also occurs with the participation of cytochrome P-450 2E1 [3]. Alcohol metabolism, ROS production, and disturbances in the redox state of cells are well-known pathways of tissue damage in several organ systems [4,5].

In rats that constantly consumed alcohol, a decrease in the activity of glutathione enzymes was observed [6]. In addition, skeletal muscles showed increased protein carbonylation [7], high levels of cholesterol hydroperoxide, and malondialdehyde (MDA) [8], indicating oxidative damage. In alcoholics, the excessive production of ROS by the microsomal system and mitochondria is noted [9]. Mitochondrial damage disrupts fatty acid oxidation and increases lipid peroxidation (LPO). Mitochondrial muscle damage was observed in 28% of alcoholics [10].

The activity of the antioxidant system plays a significant role in the pathogenesis of myopathy. Ethanol-treated rats showed an increase in superoxide dismutase (SOD) and glutathione peroxidase (GP_x) activities, as well as MDA levels, which correlated with muscle iron content and type IIb fiber atrophy [11]. Zinc treatment did not reduce muscle atrophy in animals, although it did reduce MDA levels. Zinc, manganese, copper, and selenium are cofactors of antioxidant enzymes, while iron accumulation can contribute to LPO [12]. The authors [13] found a significant decrease in the levels of alpha-tocopherol and selenium in the blood of alcoholics with skeletal myopathy. At the same time, no significant changes in the levels of such antioxidants as alpha-tocopherol, ascorbic acid, and retinol were observed in blood and muscle tissue in patients with alcoholism with myopathy [14,15].

C₆₀ fullerenes are known to be able to efficiently capture and inactivate free radicals in *in vitro* and *in vivo* systems [16,17]. C₆₀ fullerene is more powerful antioxidant than natural antioxidant vitamin E in preventing the integrity of membranes from damage [18]. Recently we demonstrated, that water-soluble C₆₀ fullerenes at low doses (0.1–1) mg kg^{−1} more effectively diminish the muscle fatigue in rats compared to the known exogenous antioxidants N-acetylcysteine and β-alanine [19,20], which are widely used in sport medicine. Moreover, they penetrate into cells and localize preferentially in mitochondria [21]. According to our previous data [22,23] water-soluble C₆₀ fullerenes at concentrations up to 14.4 as well as 24 μg ml^{−1} did not manifest any toxic effects in rat erythrocytes and thymocytes as well as in human mesenchymal stem cells, respectively. It was shown that intraperitoneal administration of C₆₀ fullerene suspension in a dose of 2.5 g kg^{−1} does not lead to mice death or to violations of their behaviour within 8 weeks [24]. It was established that the radiolabeled C₆₀ fullerene and its derivative, fullerol, after intravenous administration to mice accumulate mainly in the liver, spleen, stomach, and blood and are excreted from the body within 72 h with mainly urine [25,26]. Our recent results [27] indicate the prolonged kinetics of water-soluble C₆₀ fullerenes elimination from the body of rats, which contributes to their long-term (at least 48 h) compensatory activation of the endogenous antioxidant system in response to muscle stimulation.

In previous *in vivo* experiments, the administration of water-soluble C₆₀ fullerenes has been shown to result in significant positive therapeutic effects following ischemic injury initiation [28,29], fatigue [30], atrophy [31], and injury [32] in skeletal muscles. It is clear that the observed effects depend significantly on the doses used and the pattern of the drug administration against the background of the initiation of a particular pathology.

Thus, the aim of the present study was to evaluate the effect of water-soluble C₆₀ fullerenes as powerful exogenous antioxidants [16] on the magnitude of force response of *muscle gastrocnemius* of chronically alcoholized rats depending on the optimal dose and application pattern. In this context, it was also important to analyze biochemical indicators of blood and muscle tissue as markers of muscle damage [33], as well as histological changes of muscle tissue.

2. Materials and methods

2.1. Preparation of C₆₀FAS

C₆₀ fullerene aqueous solution (C₆₀FAS) has been prepared according to Refs. [34,35]. The proposed method is based on transferring C₆₀ molecules from the organic solution into the aqueous phase by ultrasonic treatment. The prepared C₆₀FAS is a typical colloid fluid containing single C₆₀ fullerenes (0.7 nm in diameter) as well as their nanoaggregates up to 100 nm [35,36]. The maximal concentration of C₆₀ fullerenes in water obtained by this method was 0.15 mg ml^{−1}.

The zeta potential is related to the stability of colloid dispersion. The zeta potential value for the C₆₀FAS was -25 ± 2 mV [35,37]. A high negative surface charge of the individual nanoparticles (or, more strictly, the electrostatic repulsion between the negatively charged aggregates) indicates a very low tendency for them to aggregate over time in an aqueous solution (i.e., a high solute stabilization).

The C₆₀FAS is stable within 18 months when stored at a temperature of +4 °C.

2.2. In vivo experiment

The experiments were performed on male Wistar rats aged 1–10 months (at the end of the experiment). The study protocol was approved by the Bioethics Committee of the ESC “Institute of Biology and Medicine”, Taras Shevchenko National University of Kyiv in accordance with the rules of the “European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes” and the norms of biomedical ethics in accordance with the Law of Ukraine №3447 - IV February 21, 2006, Kyiv, “The Protection of Animals from Cruelty” during biomedical research.

The control group (n = 10) received 100% drinking water. Animals in the “alcoholization” experimental group (n = 10) were randomly selected. Each animal was placed in a separate cage to receive 40% ethanol in drinking water [38], which meant that the experimental groups had no access to 100% water until the full-dosed ethanol consumption [39]. Ethanol intake was calculated as 0.5% of the animal's body weight. The ethanol dose was recalculated every 24 h during the whole experiment [40]. Duration of

alcoholization was 3, 6 and 9 months. The experimental animals of the “alcoholization + C₆₀” group (n = 10) together with alcohol orally received C₆₀FAS at a dose of 1 mg kg⁻¹ of rat weight. The C₆₀FAS dose was recalculated every 24 h throughout the experiment. The amount of C₆₀FAS administered was controlled by denying animals access to 100% drinking water until they had completely used the administered drug.

It is important to note that the selected dose of C₆₀FAS (1 mg kg⁻¹) in our experiments, as the most effective one, was chosen on the basis of previously conducted research [20,27,31]. Moreover, this dose does not present any toxicity: it is significantly lower than the LD₅₀ value, which was 600 mg kg⁻¹ body weight when administered orally to rats [41] and 721 mg kg⁻¹ when administered intraperitoneally to mice [37].

Our previous studies [19] showed that there was no significant difference in the observed effects between different routes of C₆₀FAS administration (oral and intraperitoneal). Therefore, we used in our research only the oral route of drug administration, as the most practical from the point of view of its further testing in the clinic.

The animals were anesthetized by intraperitoneal injection of nembutal (40 mg kg⁻¹). Experiment preparation included cannulation (*a. carotis communis sinistra*) for drug administration and pressure measurement, tracheotomy, and laminectomy at the level of the lumbar spinal cord. The animals were euthanized using sodium thiopental overdose, and in some cases, cervical dislocation of the cervical vertebrae was used.

2.3. Biomechanical analysis of muscle contraction

A 12-bit analog-to-digital and digital-to-analog converter (ADC-DAC) was used to record the electrophysiological signals of muscle contraction. DAC output pulses were triggered by isolated stimulators (DS2A, Digitimer), which performed nerve stimulation. The input signals were fed through the amplifier (Brownlee) to the ADC and recorded with a sampling frequency of 10 kHz. A linear motor in the servo position was used to stretch the muscle and measure the force it developed. The efforts were measured using semi-conductor strain gauges glued to rigid steel beams mounted on moving parts of the linear motor. The stiffness of the puller exceeded 0.06 N mm⁻¹, and the time constant of transient processes did not exceed 60 ms [28].

The studied *muscle gastrocnemius* was freed from the surrounding tissues, the tendon part was cut in the distal part to the lower back. To prepare for the modulated stimulation of efferents in the corresponding segments, the ventral roots were cut directly at the places of their exit from the spinal cord. The filaments of the cut ventral roots were fixed on the stimulating electrodes and the stimulus sequence was cyclically distributed. Stimulation of efferents was carried out by electrical pulses of 2 ms duration, generated by a pulse generator. External load on the muscle was controlled using a system of mechanical stimulators [28].

When analyzing the myotic response of the studied muscle, several basic biomechanical parameters were analyzed as markers of the presence of certain link dysfunctions in the “excitation-response” chain [29,31,32,42], namely:

1. Change in the level of minimum muscle contraction force;
2. Change in the time to reach the maximum force response of the muscle;
3. Change in the level of the maximum force of muscle contraction;
4. Change in the integrated power of the muscle (calculated area under the force curve);
5. Change in the time of the reduction of muscle contraction force by 50% from the initial level as the indicator of muscle fatigue development at stimulation irritations;
6. Change in the time of muscle force response to the initial level.

2.4. Biochemical analysis

The levels of enzymes (creatine phosphokinase (CPK) and lactate dehydrogenase (LDH)) as well as creatinine and lactate (LA) in the blood plasma of the test animals and assessment of the level of oxidative processes in muscle tissues (content of hydrogen peroxide and reduced glutathione (GSH) as well as catalase (CAT), selenium-dependent GP_x and SOD activities), as markers of muscle damage [33], were determined using clinical diagnostic equipment (hemoanalyzers RNL-200 (Netherlands), ABX Micros ESV60 (France) and automatic analyzer Pentra C400 (France)).

Blood alcohol concentration (BAC) was determined at the end of the experiment by cardiac puncture using an AM1 alcohol analyzer (Analox Instruments Limited, UK).

2.5. Histological analysis

The samples of *muscle gastrocnemius* were separated and fixed in 10% formalin, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin (H&E) for general histopathological analyses, or with hematoxylin and picrofuchsin by van Gieson for detecting collagen fibers [43]. The histopathological profiles of each sample were determined by light microscopy observation. Also, digital microphotographs of stained sections were taken at × 400 magnification using a computer-assisted image analyzing system (it consists of an Olympus B×41 microscope and Olympus C-5050 Zoom digital camera, Japan). Then, the muscle fiber diameters and the area occupied by connective tissue in the muscle bundles were measured using ImageJ software.

2.6. Statistical analysis

Statistical processing of the measurement results was carried out by methods of variation statistics using the Origin 9.4 software. Each of the experimental kinetic curves is the result of averaging 10 similar measurements. No less than five repeats were performed for each biochemical and morphometrical measurement. Data are expressed as the means $\pm$ SEM for each group. The differences among experimental groups were detected by one-way ANOVA followed by Bonferroni's multiple comparison test. Values of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. BAC value

The BAC value in rats after chronic ethanol consumption ranged from 147 mg dl⁻¹ (3 months of alcohol consumption) to 253 mg dl⁻¹ (9 months of alcohol consumption) (Fig. 1). These data agree well with the results of a study [39].

C₆₀FAS administration at a dose of 1 mg kg⁻¹ in the therapeutic scheme (together with alcohol) did not result in a significant change in the BAC values throughout the experiment (Fig. 1).

3.2. Muscle contraction force analysis

Fig. 2 shows the mechanograms of the 1st and 10th *muscle gastrocnemius* contraction in alcoholized rats at 50 Hz for 6 s stimulation. The recording of force responses reveals a sharp decrease in muscle force activity already at the 1st stimulus with a progressive decrease to (50–70)% of control values at the 10th one. A clear decrease in the amplitude of *muscle gastrocnemius* contraction in alcoholic rats was observed with increasing time of alcoholization from 3 to 6 and 9 months. In rats receiving alcohol and C₆₀FAS (1 mg kg⁻¹) together, an increase in the muscle force response was observed throughout the experiment compared to rats in the alcoholization group.

For a more qualitative analysis of the findings, we analyzed changes in the main biomechanical markers of muscle activity (Fig. 3).

Integrated muscle power, as an overall muscle performance indicator, is one of the most important characteristics of muscle dysfunction. With 3, 6 and 9 months of alcoholization, it decreased at the 1st contraction by 22 $\pm$ 2%, 29 $\pm$ 1%, and 34 $\pm$ 3%, respectively. It should be noted that at the 10th contraction, this parameter decreased by 34 $\pm$ 3%, 56 $\pm$ 5%, and 74 $\pm$ 4%, respectively, compared to the 1st contraction (Fig. 3a). C₆₀FAS administration resulted in an 11 $\pm$ 1%, 18 $\pm$ 1%, and 26 $\pm$ 2% increase in integrated muscle power at 3, 6 and 9 months of alcoholization, respectively. The difference between the 1st and 10th contractions was reduced to (15–39)%.

A change in minimum contraction force is one of the most sensitive markers of muscle dysfunction. Its decrease at the 1st contraction was 35 $\pm$ 3%, 56 $\pm$ 3%, and 68 $\pm$ 5% with alcoholization of 3, 6 and 9 months, respectively (Fig. 3b). At the 10th contraction, this parameter decreased by 56 $\pm$ 3%, 78 $\pm$ 4% and 91 $\pm$ 5% from the control values, indicating severe impairment of the studied muscle function. C₆₀FAS administration increased this parameter by 9 $\pm$ 1%, 26 $\pm$ 2%, and 33 $\pm$ 3% with 3, 6 and 9 months of alcoholization, respectively.

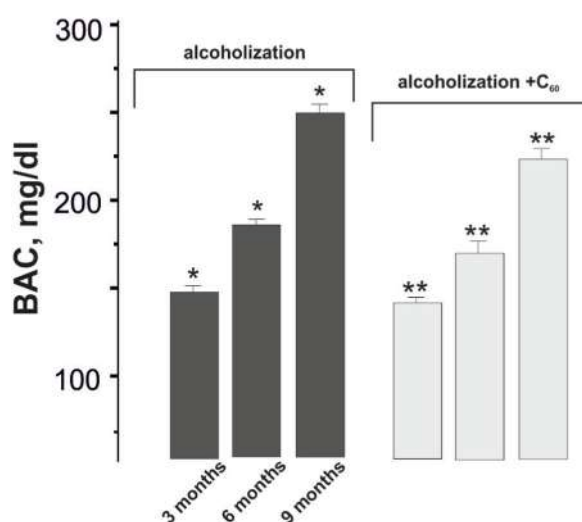


Fig. 1. Blood alcohol concentration (BAC) in rats after chronic ethanol administration: alcoholization - rats treated with alcohol; alcoholization + C₆₀ - rats treated with a mixture of alcohol and C₆₀FAS at a dose of 1 mg kg⁻¹ during the whole period of alcoholization; 3, 6 and 9 months - alcoholization lasting 3, 6 and 9 months, respectively. * $p < 0.05$; ** $p < 0.05$ relative to the alcoholization group.

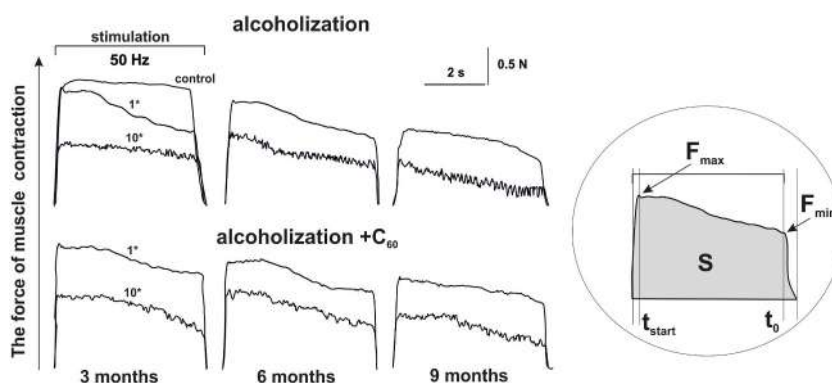


Fig. 2. The forces of the 1st (1*) and 10th (10*) contractions of the *muscle gastrocnemius* of alcoholized rats induced by 6 s non-relaxation stimulation pools at 50 Hz: alcoholization - rats treated with alcohol; alcoholization + C₆₀ - rats treated with a mixture of alcohol and C₆₀FAS at a dose of 1 mg kg⁻¹ during the whole period of alcoholization; 3, 6 and 9 months - alcoholization lasting 3, 6 and 9 months, respectively; F_{max} and F_{min} - the maximum and minimum levels of muscle force response; S - the integrated power of the muscle (calculated area under the force curve); t_{start} - the time of force reaching its maximum value; t₀ - the time of force response reaching the initial level.

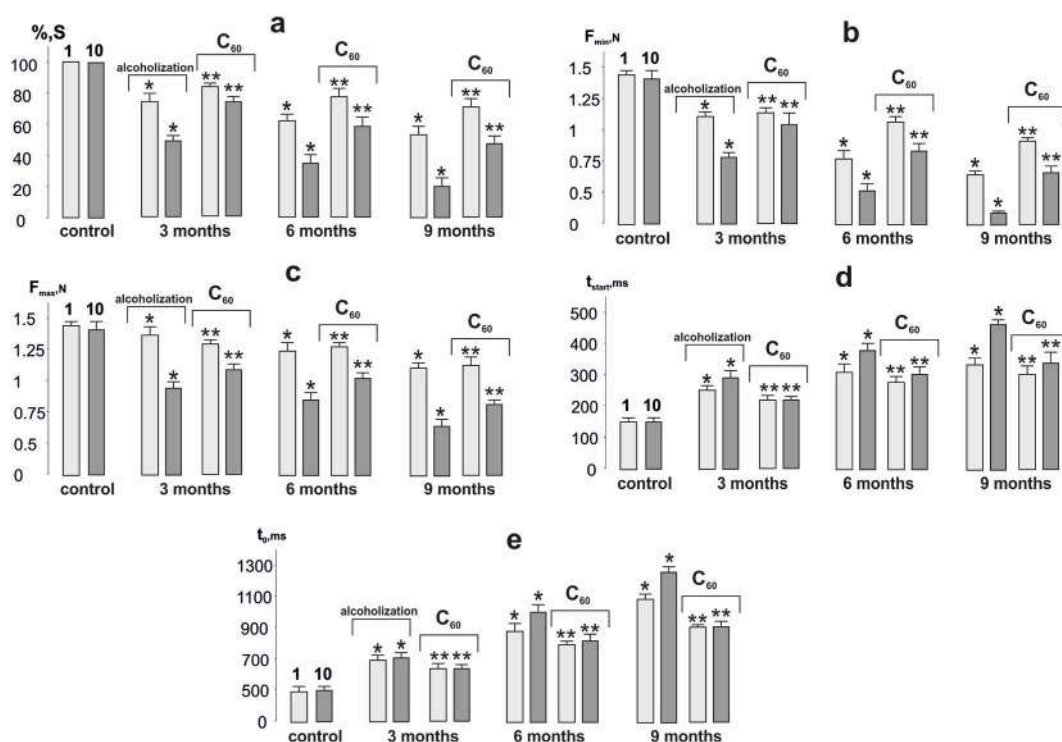


Fig. 3. The levels of biomechanical markers of *muscle gastrocnemius* contraction in alcoholized rats when applied 6 s with non-relaxation stimulation at 50 Hz: alcoholization - rats treated with alcohol; C₆₀ - rats treated with a mixture of alcohol and C₆₀FAS at a dose of 1 mg kg⁻¹ during the whole period of alcoholization; 1 and 10 - the strength of the 1st and 10th contractions, respectively; 3, 6 and 9 months - alcoholization lasting 3, 6 and 9 months, respectively; S - integrated muscle power (presented as a percentage of the maximum values) (a); F_{min} and F_{max} - minimum (b) and maximum (c) levels of muscle strength response; t_{start} - time of force reaching its maximum value (d); t₀ - time of force response reaching initial level (e); *p < 0.05 relative to the control; **p < 0.05 relative to the alcoholization group.

A change in maximum contraction force levels is an indicator of overall muscle system dysfunction, indicating a reduction in the maximum possible force response during the development of pathology (Fig. 3c). At the 1st contraction this index decreased slightly, namely by 8 ± 1%, 12 ± 1% and 21 ± 2%, with 3, 6 and 9 months alcoholization, respectively. However, at the 10th contraction, this reduction was 47 ± 2%, 52 ± 3%, and 64 ± 5%, respectively, indicating a significant dysfunction of the maximal muscle force. The increase in maximal force contraction with C₆₀FAS administration was 11 ± 1%, 17 ± 1%, and 23 ± 2% with 3, 6 and 9 months of alcoholization, respectively.

The increase in the time to maximum force response indicates the level of physiological dysfunction of the muscle apparatus when it implements its maximum force tasks during precise positional movements (Fig. 3d). Its increase at 3, 6 and 9 months of alcoholization was $31 \pm 3\%$, $44 \pm 4\%$ and $49 \pm 4\%$, respectively, at the 1st contraction with a further increase of (25–30)% at the 10th one. The correction for this parameter with C₆₀FAS administration was (15–20)%. Note that in this case the difference between the 1st and 10th contractions was reduced to (5–8)%.

The change in muscle force response release time to the initial level is associated with an increase in intramuscular collagen structures, an increase in subfascial pressure and the presence of non-functioning muscle fibers. The studied parameter describes a change in muscle stiffness associated with both an increase in connective tissue components of the muscle (long-term pathology) and a change in intramuscular pressure (acute period of pathology). Its increase was $48 \pm 4\%$, $88 \pm 8\%$, and $123 \pm 8\%$ with 3, 6 and 9 months of alcoholization, respectively, at the 1st contraction, with a (9–12)% increase at the 10th one. C₆₀FAS administration reduced this parameter by $15 \pm 1\%$, $29 \pm 2\%$, and $41 \pm 3\%$, respectively. The difference between the 1st and 10th contractions was reduced to (3–5)%.

Thus, the proposed therapeutic regimen of C₆₀FAS administration (1 mg kg^{-1} ; together with alcohol) leads to significant positive biomechanical changes in the *muscle gastrocnemius* contractile processes, namely, the level of muscle damage severity is reduced by an average of (35–40)%.

Recording the contractile force of rat *muscle gastrocnemius* after 3, 6 and 9 months of alcoholic induction with 1 Hz fatigue stimulation for 1800 s (Fig. 4a) showed a decrease in integrated muscle power of $82 \pm 5\%$, $60 \pm 3\%$, and $22 \pm 1\%$ of control values (Fig. 4b). Administration of C₆₀FAS to experimental animals changed this to $94 \pm 4\%$, $85 \pm 2\%$, and $69 \pm 5\%$ (Fig. 4b). The 50% reduction in force response time from initial values (t_{50}) was $1740 \pm 25 \text{ ms}$, $1460 \pm 91 \text{ ms}$, and $1220 \pm 25 \text{ ms}$ (Fig. 4c). When C₆₀FAS was administered to rats subjected to 3 and 6 months of alcoholization, no 50% reduction in force response was observed (Fig. 4c). With alcoholization lasting 9 months, this parameter was $1620 \pm 28 \text{ ms}$. Thus, it can be argued that the proposed therapeutic regimen of C₆₀FAS administration (dose 1 mg kg^{-1} ; together with alcohol) reduces the time of occurrence of fatigue processes in alcohol-induced muscle and is most effective specifically in the long-term development of alcoholic myopathy.

The results obtained indicate that the consequence of alcoholic myopathy is the deterioration of contractile muscle activity. The reason for this is both ultrastructural changes of myocytes and their atrophy, and changes in electrolyte homeostasis and bioelectric activity in general [44]. During the development of alcoholic myopathy, first of all, there is a hypergeneration of free radicals in dysfunctional mitochondria [45,46], which causes the destruction of the membrane structures of myocytes and functional disorders of their enzyme systems [47]: a decrease in the activity of Na^+/K^+ -ATPase and an increase in $-\text{Ca}^{2+}$ -ATPase. The mitotic disorders cause an imbalance in the correction of the joints positioning and progressive muscle weakness, which leads to the degradation of muscle strength with 3, 6 and 9 months of alcoholization. From a functional point of view, this indicates that a large amount of high-energy phosphate compounds is consumed by the damaged muscle cell to maintain homeostasis and, as a consequence, there is a metabolic disturbance, leading to a significant increase in muscle fatigue with the development of alcoholic myopathy. At the same time, the use

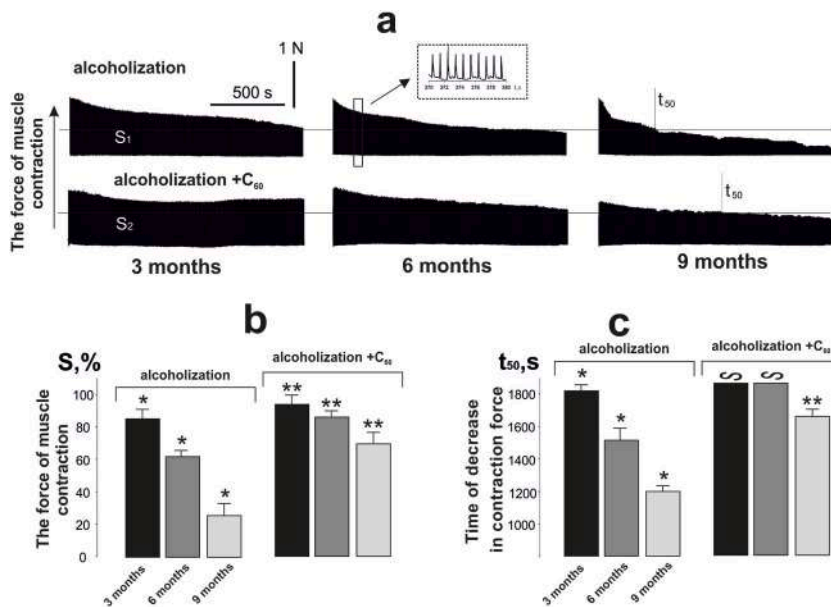


Fig. 4. *Muscle gastrocnemius* contraction force of alcoholized rats induced by 1 Hz relaxation-free stimulation lasting 1800 s (a), and biomechanical parameters of fatigue development: S - integrated muscle power (b); time to 50% decrease in force response from initial values (t_{50}) (c); alcoholization - rats treated with alcohol; alcoholization + C₆₀ - rats treated with a mixture of alcohol and C₆₀FAS at a dose of 1 mg kg^{-1} during the whole period of alcoholization; 3, 6 and 9 months - alcoholization lasting 3, 6 and 9 months, respectively; * $p < 0.05$; ** $p < 0.05$ relative to the alcoholization group.

of C₆₀FAS significantly increases the energy capacity of actively contracting alcohol-induced muscle.

3.3. The analysis of biochemical parameters in blood plasma and muscle tissue

To confirm the biomechanical results obtained, we analyzed blood plasma biochemical indices, which are used to diagnose the development of muscle myopathies [33]. In particular, changes in creatinine, CPK, LA, and LDH levels made it possible to assess the physiological state of *muscle gastrocnemius* against the background of prolonged alcoholization of animals (Fig. 5).

It should be noted that the above biochemical markers have a pronounced tendency to increase with the increasing duration of alcoholization of animals. In cases where rats received a mixture of alcohol and C₆₀FAS, these indices were significantly lower compared to alcohol-impaired animals. Thus, creatinine was reduced by $16 \pm 1\%$, $18 \pm 1\%$, and $37 \pm 2\%$, CPK by $13 \pm 1\%$, $18 \pm 1\%$, and $28 \pm 2\%$, LDH by $15 \pm 1\%$, $29 \pm 2\%$ and $41 \pm 2\%$, and LA by $19 \pm 1\%$, $27 \pm 2\%$ and $38 \pm 2\%$ when alcohol and C₆₀FAS were co-administered and alcoholization lasted 3, 6 and 9 months, respectively (Fig. 5a). Thus, the observed changes in biochemical indicators

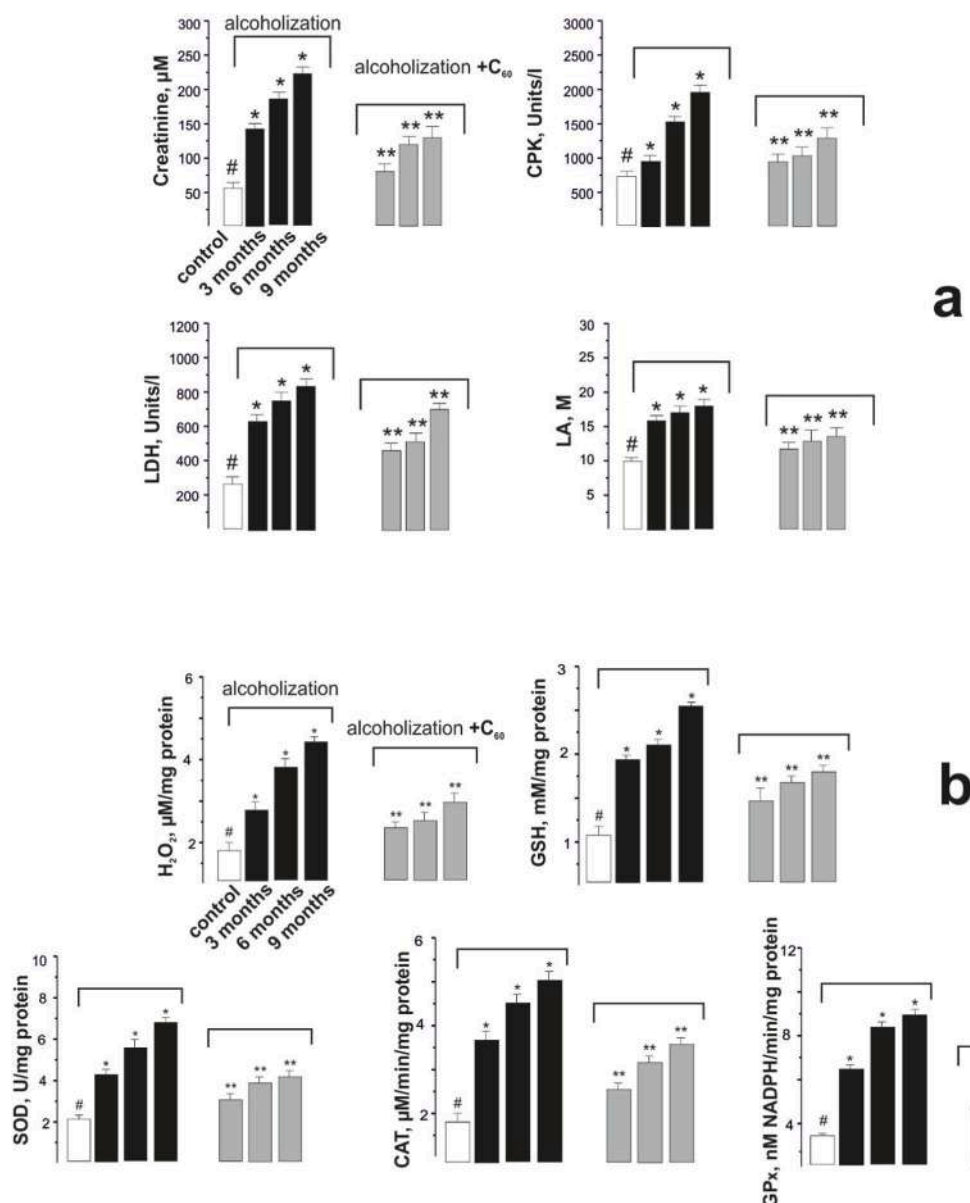


Fig. 5. The content of creatinine, CPK, LDH, and LA in the blood plasma of alcoholized rats (a) and indicators of pro- and antioxidant balance (H₂O₂, GSH, SOD, CAT, and GPx) in rat *muscle gastrocnemius* (b): alcoholization - rats treated with alcohol; alcoholization + C₆₀ - rats treated with a mixture of alcohol and C₆₀FAS at a dose of 1 mg kg⁻¹ during the whole period of alcoholization; 3, 6 and 9 months - alcoholization lasting 3, 6 and 9 months, respectively; **p* < 0.05 relative to the control; ***p* < 0.05 relative to the alcoholization group.

in the blood plasma of tested animals correlate well with the biomechanical parameters of *muscle gastrocnemius* functioning described above.

The obtained positive therapeutic effect of water-soluble C₆₀ fullerenes can be explained by their powerful antioxidant properties [19,41]. The determination of changes in the pro- and antioxidant balance in the tissues of the muscle under study is essential to confirm this hypothesis [48].

H₂O₂ concentration increased with the increasing duration of alcoholization and was $151 \pm 7\%$, $327 \pm 9\%$, and $414 \pm 11\%$ when alcoholization lasted 3, 6 and 9 months, respectively, compared with the control muscle. Co-administration of alcohol and C₆₀FAS resulted in $28 \pm 2\%$, $44 \pm 2\%$, and $51 \pm 2\%$ reductions in H₂O₂ and GSH by $13 \pm 1\%$, $27 \pm 1\%$, and $42 \pm 2\%$, activities of SOD by $21 \pm 1\%$, $29 \pm 1\%$ and $44 \pm 2\%$, CAT by $12 \pm 1\%$, $34 \pm 2\%$ and $48 \pm 2\%$, and GP_x by $17 \pm 1\%$, $23 \pm 1\%$ and $39 \pm 2\%$ during alcoholization of 3, 6 and 9 months, respectively (Fig. 5b). It is important to note that the increase in muscle GSH content due to C₆₀FAS increases resistance to exercise [30]. Thus, in all tests there is a positive change in the pro- and antioxidant biochemical balance in rat *muscle gastrocnemius* by about (15–30)% when C₆₀FAS is administered at a dose of 1 mg kg^{-1} , together with alcohol.

Thus, based on the data obtained, it can be concluded that the application of water-soluble C₆₀ fullerenes contributes to the reduction of oxidative processes in muscle by maintaining the balance between pro-oxidants and antioxidant defense system, prevents the negative effect of ROS on cellular and subcellular structures in the development of alcoholic myopathy in rats. In our opinion, C₆₀ fullerene affects the activity of endogenous antioxidants, suppressing the occurrence of destruction in muscle and thus reducing its degradation.

3.4. Histological analysis of muscle tissue

In alcoholized rats, most of the muscle fibers are thinner than in the control group (Fig. 6a and d), so the average diameter of the fibers is reduced to $24.4 \mu\text{m}$ (Table 1). There are tortuous fibers. Some of the fibers have an uneven thickness along the fiber. Some fibers are vacuolized, which indicates destructive processes in them. In some fibers, the transverse striation is less clear, which may indicate a violation of the correct spatial arrangement of actin-myosin complexes. Within thin fibers, there are also fibers of normal thickness (Fig. 6b). Connective tissue between muscle fibers is enlarged (Fig. 6e). As a result, the area occupied by connective tissue increase up to $0.21 \mu\text{m}^2$ per $1 \mu\text{m}^2$ cross-sectional area of the muscle (Table 1). So, sclerosis and fibrosis are observed.

In alcoholized rats that received C₆₀FAS, the thickness of the muscle fibers is not reduced compared to the control group. Sclerosis and fibrosis are lesser. So, the pathohistological manifestations in this group of rats are smaller (Table 1; Fig. 6c and f).

Summarizing, the following mechanism of C₆₀ fullerene action can be considered. It is known that more than 90% of consumed alcohol is metabolized by oxidative and non-oxidative pathways, producing such chemically active compounds as acetaldehyde, acetate, fatty acid ethyl ester, etc. [49]. These compounds generate ROS, which cause increased oxidative stress and LPO, disrupting thus the structural integrity of cells and tissue functions in general. Therefore, C₆₀ fullerenes, as powerful antioxidants [16,17], effectively absorbing ROS, normalize the functional state of the muscular system against the background of alcoholic myopathy.

4. Conclusions

Thus, the work demonstrated for the first time in animal models that the level of chronic alcoholic myopathy development decreased when water-soluble C₆₀ fullerene at a dose of 1 mg kg^{-1} was used as a therapeutic nanoagent, which was confirmed by the biomechanical markers analysis of the contractile process of *muscle gastrocnemius*. The proposed combined administration of alcohol and C₆₀FAS leads to a decrease in the time of fatigue processes in alcohol-induced muscle and is most effective precisely in the long-term development of alcoholic myopathy. The analysis of blood plasma biochemical parameters, changes in pro- and antioxidant balance, and histological indices in the tissues of the studied *muscle gastrocnemius* make it possible to state that the administration of water-soluble C₆₀ fullerenes as potent antioxidants prevents the negative effects of ROS on cellular and subcellular structures in alcoholic myopathy development. This opens up new possibilities in the therapy of the pathological conditions of the muscular system, which are based on the pathological action of free-radical processes.

Author contribution statement

Olexandr Motuziuk, Dmytro Nozdrenko, Svitlana Prylutska, Igor Vareniuk, Kateryna Bogutska, Serhii Braniuk, Olexandr Korotkyi, Yuriy Prylutskyi, Uwe Ritter and Jacek Piosik: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

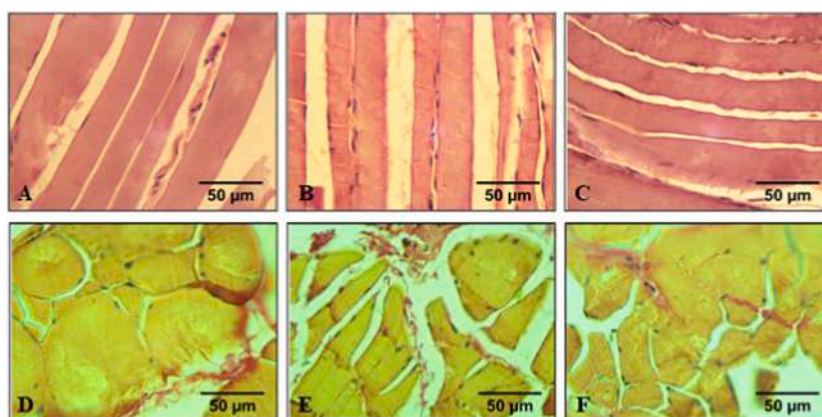


Fig. 6. Representative histological images of *muscle gastrocnemius*: a and d - control group; b and e – alcoholization group; c and f - alcoholization + C₆₀ group. a,b and c – hematoxylin and eosin staining; d,e and f - hematoxylin and picrofuchsin staining by van Gieson. Scale bar - 50 µm.

Table 1

Morphometrical data of *muscle gastrocnemius* in rats after alcohol and C₆₀FAS consumption.

Group	Diameter of muscle fibers, µm	Area occupied by connective tissue, µm ² /µm ²
Control	36.7 ± 1.3	0.15 ± 0.02
Alcoholization	24.4 ± 1.4*	0.21 ± 0.02*
Alcoholization + C ₆₀	32.8 ± 2.5**	0.17 ± 0.02**

p* < 0.05 compared with control; *p* < 0.05 compared with alcoholization group.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We acknowledge support for the publication costs by the Open Access Publication Fund of the University of Gdansk, Poland.

References

- [1] T.L. Nemirovskaya, B.S. Shenkman, O.E. Zinovyeva, YuN. Kazantseva, N.D. Samkhaeva, Development of clinical and morphological signs of chronic alcoholic myopathy in men with prolonged alcohol intoxication, *Fiziol. Chel.* 41 (2015) 625–629.
- [2] B. Halliwell, M. Whiteman, Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br. J. Pharmacol.* 142 (2004) 231–255, <https://doi.org/10.1038/sj.bjp.0705776>.
- [3] A.I. Cederbaum, Introduction-serial review: alcohol, oxidative stress and cell injury, *Free Radic. Biol. Med.* 31 (2001) 1524–1526, [https://doi.org/10.1016/S0891-5849\(01\)00741-9](https://doi.org/10.1016/S0891-5849(01)00741-9).
- [4] J. Fernández-Solà, V.R. Preedy, C.H. Lang, E. Gonzalez-Reimers, M. Arno, J.C.I. Lin, H. Wiseman, S. Zhou, P.W. Emery, T. Nakahara, K. Hashimoto, M. Hirano, F. Santolaria-Fernández, T. González-Hernández, F. Fatjó, E. Sacanella, R. Estruch, J.M. Nicolás, A. Urbano-Márquez, Molecular and cellular events in alcohol-induced muscle disease, *Alcohol Clin. Exp. Res.* 31 (2007) 1953, <https://doi.org/10.1111/j.1530-0277.2007.00530.x>, 1562.
- [5] P.E. Molina, J.D. Gardner, F.M. Souza-Smith, A.M. Whitaker, Alcohol abuse: critical pathophysiological processes and contribution to disease burden, *Physiology* 29 (2014) 203–215, <https://doi.org/10.1152/physiol.00055.2013>.
- [6] J.S. Otis, L.A. Brown, D.M. Guidot, Oxidant-induced atrogen-1 and transforming growth factor-beta1 precede alcohol-related myopathy in rats, *Muscle Nerve* 36 (2007) 842–848, [https://doi.org/10.1016/s0304-3835\(02\)00006-x](https://doi.org/10.1016/s0304-3835(02)00006-x).
- [7] G.J. Dekeyser, C.R. Clary, J.S. Otis, Chronic alcohol ingestion delays skeletal muscle regeneration following injury, *Regen. Med. Res.* 1 (2013) 2.
- [8] M.C. Gomes-Marcondes, M.J. Tisdale, Induction of protein catabolism and the ubiquitin-proteasome pathway by mild oxidative stress, *Cancer Lett.* 180 (2002) 69–74, [https://doi.org/10.1016/S0304-3835\(02\)00006-x](https://doi.org/10.1016/S0304-3835(02)00006-x).
- [9] N.S. Aberle, J. Ren, Short-term acetaldehyde exposure depresses ventricular myocyte contraction: role of cytochrome P450 oxidase, xanthine oxidase, and lipid peroxidation, *Alcohol Clin. Exp. Res.* 27 (2003) 577–583, <https://doi.org/10.1097/01.ALC.0000060522.40447.8E>.
- [10] J.C. Romero, F. Santolaria, E. González-Reimers, L. Díaz-Flores, A. Conde, F. Rodríguez-Moreno, N. Batista, Chronic alcoholic myopathy and nutritional status, *Alcohol* 11 (1994) 549–555, [https://doi.org/10.1016/0741-8329\(94\)90082-5](https://doi.org/10.1016/0741-8329(94)90082-5).
- [11] M.C. Durán Castellón, E. González-Reimers, A. López-Lirola, R. Martín Olivera, F. Santolaria-Fernández, L. Galindo-Martín, P. Abreu-González, T. González-Hernández, Alcoholic myopathy: lack of effect of zinc supplementation, *Food Chem. Toxicol.* 43 (2005) 1333–1343, <https://doi.org/10.1016/j.fct.2005.03.006>.
- [12] G.A. Ramm, R.G. Ruddell, Hepatotoxicity of iron overload: mechanisms of iron-induced hepatic fibrogenesis, *Semin. Liver Dis.* 25 (2005) 433–449, <https://doi.org/10.1055/s-2005-923315>.
- [13] R.J. Ward, T.J. Peters, The antioxidant status of patients with either alcohol-induced liver damage or myopathy, *Alcohol* 27 (1992) 359–365.
- [14] J. Fernández-Solà, E. Villegas, J. M. Nicolás, R. Deulofeu, E. Antúnez, E. Sacanella, R. Estruch, A. Urbano-Márquez, Serum and muscle levels of alpha-tocopherol, ascorbic acid, and retinol are normal in chronic alcoholic myopathy, *Alcohol Clin. Exp. Res.* 22 (1998) 422–427.

- [15] J. Fernández-Solà, G. García, M. Elena, E. Tobías, E. Sacanella, R. Estruch, J.-M. Nicolás, Muscle antioxidant status in chronic alcoholism, *Alcohol Clin. Exp. Res.* 26 (2002) 1858–1862.
- [16] S.V. Eswaran, Water soluble nanocarbon materials: a panacea for all? *Curr. Sci.* 114 (2018) 1846–1850, <https://doi.org/10.18520/cs/v114/i09/1846-1850>.
- [17] C.A. Ferreira, D. Ni, Z.T. Rosenkrans, W. Cai, Scavenging of reactive oxygen and nitrogen species with nanomaterials, *Nano Res.* 11 (2018) 4955–4984, <https://doi.org/10.1007/s12274-018-2092-y>.
- [18] I.C. Wang, L.A. Tai, D.D. Lee, P.P. Kanakamma, C.K. Shen, T.Y. Luh, C.H. Cheng, K.C. Hwang, C(60) and water-soluble fullerene derivatives as antioxidants against radical-initiated lipid peroxidation, *J. Med. Chem.* 22 (1999) 4614–4620, <https://doi.org/10.1021/jm990144s>.
- [19] I.V. Vereshchaka, N.V. Bulgakova, A.V. Maznychenko, O.O. Gonchar, Yul. Prylutsky, U. Ritter, W. Moska, T. Tomiak, D.M. Nozdrenko, I.V. Mishchenko, A. I. Kostyukov, C₆₀ fullerenes diminish muscle fatigue in rats comparable to N-acetylcysteine or β-alanine, *Front. Physiol.* 9 (2018) 517, <https://doi.org/10.3389/fphys.2018.00517>.
- [20] D. Nozdrenko, S. Prylutska, K. Bogutska, V. Cherepanov, A. Senenko, O. Vygovska, S. Khrapaty, U. Ritter, Yu Prylutsky, J. Piosik, Analysis of biomechanical and biochemical markers of rat *muscle soleus* fatigue processes development during long-term use of C₆₀ fullerene and N-acetylcysteine, *Nanomaterials* 12 (2022) 1552, <https://doi.org/10.3390/nano12091552>.
- [21] A. Grebinyk, S. Grebinyk, S. Prylutska, U. Ritter, O. Matyshevska, T. Dandekar, M. Frohme, C₆₀ fullerene accumulation in human leukemic cells and perspectives of LED-mediated photodynamic therapy, *Free Radic. Biol. Med.* 124 (2018) 319–327, <https://doi.org/10.1016/j.freeradbiomed.2018.06.022>.
- [22] S.V. Prylutska, I.I. Grynyuk, S.M. Grebinyk, O.P. Matyshevska, Yul. Prylutsky, U. Ritter, C. Siegmund, P. Scharff, Comparative study of biological action of fullerenes C₆₀ and carbon nanotubes in thymus cells, *Mat-wiss. u. Werkstofftech.* 40 (2009) 238–241, <https://doi.org/10.1002/mawe.200900433>.
- [23] M. Tolkachov, V. Sokolova, K. Loza, V. Korolovych, Y. Prylutsky, M. Eppe, U. Ritter, P. Scharff, Study of biocompatibility effect of nanocarbon particles on various cell types *in vitro*, *Mat-wiss. u. Werkstofftech.* 47 (2016) 216–221, <https://doi.org/10.1002/mawe.201600486>.
- [24] F. Moussa, F. Trivin, R. Ceolin, M. Hadchouel, P.Y. Sizaret, V. Greugny, C. Fabre, A. Rassat, H. Szwarc, Early effects of C₆₀ administration in Swiss mice: a preliminary account for *in vivo* C₆₀ toxicity, *Fullerene Sci. Technol.* 4 (1996) 21–29, <https://doi.org/10.1080/10641229608001534>.
- [25] Z.Q. Ji, H. Sun, H. Wang, Q. Xie, Y. Liu, Z. Wang, Biodistribution and tumor uptake of C₆₀(OH)_x in mice, *J. Nanopart. Res.* 8 (2006) 53–63, <https://doi.org/10.1007/s11051-005-9001-5>.
- [26] N. Nikolic, S. Vranjes-Ethurić, D. Janković, D. Ethokic, M. Mirković, N. Bibić, V. Trajković, Preparation and biodistribution of radiolabeled fullerene C₆₀ nanocrystals, *Nanotechnol* 20 (2009), 385102, <https://doi.org/10.1088/0957-4484/20/38/385102>.
- [27] Yu Prylutsky, D. Nozdrenko, O. Gonchar, S. Prylutska, K. Bogutska, E. Täuscher, P. Scharff, U. Ritter, The residual effect of C₆₀ fullerene on biomechanical and biochemical markers of the *muscle soleus* fatigue development in rats, *J. Nanomater.* 2023 (2023), 2237574, <https://doi.org/10.1155/2023/2237574>.
- [28] D.M. Nozdrenko, D.O. Zavadovskiy, T.Yu Matvienko, S.Yu Zay, K.I. Bogutska, Yul. Prylutsky, U. Ritter, P. Scharff, C₆₀ fullerene as promising therapeutic agent for the prevention and correction of skeletal muscle functioning at ischemic injury, *Nanoscale Res. Lett.* 12 (2017) 115, <https://doi.org/10.1186/s11671-017-1876-4>.
- [29] D. Nozdrenko, T. Matvienko, O. Vygovska, K. Bogutska, O. Motuziuk, N. Nurishchenko, Yu Prylutsky, P. Scharff, U. Ritter, Protective effect of water-soluble C₆₀ fullerene nanoparticles on the ischemia-reperfusion injury of the *muscle soleus* in rats, *Int. J. Mol. Sci.* 22 (2021) 6812, <https://doi.org/10.3390/ijms22136812>.
- [30] Yul. Prylutsky, I.V. Vereshchaka, A.V. Maznychenko, N.V. Bulgakova, O.O. Gonchar, O.A. Kyzyma, U. Ritter, P. Scharff, T. Tomiak, D.M. Nozdrenko, I. V. Mishchenko, A.I. Kostyukov, C₆₀ fullerene as promising therapeutic agent for correcting and preventing skeletal muscle fatigue, *J. Nanobiotechnol.* 15 (2017) 8, <https://doi.org/10.1186/s12951-016-0246-1>.
- [31] D. Nozdrenko, S. Prylutska, K. Bogutska, N. Nurishchenko, O. Abramchuk, O. Motuziuk, Y. Prylutsky, P. Scharff, U. Ritter, Effect of C₆₀ fullerene on recovery of *muscle soleus* in rats after atrophy induced by achillotenotomy, *Life* 12 (2022) 332, <https://doi.org/10.3390/life12030332>.
- [32] D. Nozdrenko, T. Matvienko, O. Vygovska, V. Soroca, K. Bogutska, A. Zholos, P. Scharff, U. Ritter, Y. Prylutsky, Post-traumatic recovery of *muscle soleus* in rats is improved via synergistic effect of C₆₀ fullerene and TRPM8 agonist menthol, *Appl. Nanosci.* 12 (2022) 467–478, <https://doi.org/10.1007/s13204-021-01703-z>.
- [33] P. Brancaccio, G. Lippi, N. Maffulli, Biochemical markers of muscular damage, *Clin. Chem. Lab. Med.* 48 (2010) 757–767, <https://doi.org/10.1515/CCLM.2010.179>.
- [34] Yul. Prylutsky, V.M. Yashchuk, K.M. Kushnir, A.A. Golub, V.A. Kudrenko, S.V. Prylutska, I.I. Grynyuk, E.V. Buzaneva, P. Scharff, T. Braun, O.P. Matyshevska, Biophysical studies of fullerene-based composite for bio-nanotechnology, *Mater. Sci. Eng. C* 23 (2023) 109–111.
- [35] U. Ritter, Yul. Prylutsky, M.P. Evstigneev, N.A. Davidenko, V.V. Cherepanov, A.I. Senenko, O.A. Marchenko, A.G. Naumovets, Structural features of highly stable reproducible C₆₀ fullerene aqueous colloid solution probed by various techniques, *Fullerenes, Nanotub. Carbon Nanostruct.* 23 (2015) 530–534, <https://doi.org/10.1080/1536383X.2013.870900>.
- [36] G.B. Skamrova, I.V. Laponogov, A.S. Buchelnikov, Y.G. Shchekorbatov, S.V. Prylutska, U. Ritter, Y.I. Prylutsky, M.P. Evstigneev, Interceptor effect of C₆₀ fullerene on the *in vitro* action of aromatic drug molecules, *Eur. Biophys. J.* 43 (2014) 265–276, <https://doi.org/10.1007/s00249-014-0960-2>.
- [37] S.V. Prylutska, A.G. Grebinyk, O.V. Lynchak, I.V. Byelinska, V.V. Cherepanov, E. Tauscher, O.P. Matyshevska, Yu I. Prylutsky, V.K. Rybalchenko, U. Ritter, M. Frohme, *In vitro* and *in vivo* toxicity of pristine C₆₀ fullerene aqueous colloid solution, *Fullerenes, Nanotub. Carbon Nanostruct.* 27 (2019) 715–728, <https://doi.org/10.1080/1536383X.2019.1634055>.
- [38] M.A. Collins, E.J. Neasey, Alcohol, excitotoxicity and adult brain damage: an experimentally unproven chain-of-events, *Front. Mol. Neurosci.* 9 (2016) 8, <https://doi.org/10.3389/fnmol.2016.00008>.
- [39] K. Song, R.A. Coleman, X. Zhu, C. Alber, Z.K. Ballas, T.J. Waldschmidt, R.T. Cook, Chronic ethanol consumption by mice results in activated splenic T cells, *J. Leukoc. Biol.* 72 (2002) 1109–1116.
- [40] N.B. D'Souza El-Guindy, E.J. Kovacs, P. De Witte, C. Spies, J.M. Littleton, W.J.S. De Villiers, A.J. Lott, T.P. Plackett, N. Lanzke, G.G. Meadows, Laboratory models available to study alcohol-induced organ damage and immune variations: choosing the appropriate model, *Alcohol Clin. Exp. Res.* 34 (2010) 1489–1551, <https://doi.org/10.1111/j.1530-0277.2010.01234.x>.
- [41] N. Gharbi, M. Pressac, M. Hadchouel, H. Szwarc, S.R. Wilson, F. Moussa, [60]fullerene is a powerful antioxidant *in vivo* with no acute or subacute toxicity, *Nano Lett.* 5 (2005) 2578–2585, <https://doi.org/10.1021/nl051866b>.
- [42] Y. Prylutsky, D. Nozdrenko, O. Gonchar, S. Prylutska, K. Bogutska, D. Franskevych, B. Hromovych, P. Scharff, U. Ritter, C₆₀ fullerene attenuates muscle force reduction in a rat during fatigue development, *Heliyon* 8 (2022), e12449, <https://doi.org/10.1016/j.heliyon.2022.e12449>.
- [43] S.K. Suvarna, C. Layton, J.D. Bancroft, *Bancroft's Theory and Practice of Histological Techniques*, seventh ed., Elsevier, 2013, p. 603.
- [44] A. Kumar, G. Davuluri, N. Welch, A. Kim, M. Gangadhariah, A. Allawy, A. Priyadarshini, M.R. McMullen, Y. Sandler, B. Willard, C.L. Hoppel, L.E. Nagy, S. Dasarthy, Oxidative stress mediates ethanol-induced skeletal muscle mitochondrial dysfunction and dysregulated protein synthesis and autophagy, *Free Radic. Biol. Med.* 145 (2019) 284–299, <https://doi.org/10.1016/j.freeradbiomed.2019.09.031>.
- [45] A.M. Pruznak, J. Nystrom, C.H. Lang, Direct central nervous system effect of alcohol alters synthesis and degradation of skeletal muscle protein, *Alcohol Alcohol* 48 (2013) 138–145, <https://doi.org/10.1093/alcalc/ags113>.
- [46] N.A. Osna, M.N. Aaron, R.S. Dagur, P. Thomes, L. Simon, D. Levitt, P. McTernan, P.E. Molina, H.Y. Choi, K. Machida, K.E. Sherman, A. Riva, S. Phillips, S. Chokshi, K.K. Kharbanda, S. Weinman, M. Ganesan, A review of alcohol–pathogen interactions: new insights into combined disease pathomechanisms, *Alcohol Clin. Exp. Res.* 46 (2022) 359–370, <https://doi.org/10.1111/acer.14777>.
- [47] J. Alleyne, A.M. Dopic, Alcohol use disorders and their harmful effects on the contractility of skeletal, cardiac and smooth muscles, *Adv. Drug Alcohol Res.* 1 (2021), 10011, <https://doi.org/10.3389/ADAR.2021.10011>.
- [48] K. Suzuki, H. Ohno, S. Oh-ishi, T. Kizaki, T. Ookawara, J. Fukii, A. Radak, N. Taniguchi, Superoxide dismutases in exercise and disease, in: C.K. Sen, L. Packer, O. Hanninen (Eds.), *Handbook of Oxidants and Antioxidants and Exercise*, Elsevier, Amsterdam, 2000, pp. 243–295.
- [49] M.A. Schukit, Ethanol and methanol, in: Goodman and Gillman's *Pharmacological Basis of Therapeutics*, twelfth ed., McGraw Hill, New Delhi, 2011, pp. 629–647.



C₆₀ fullerene reduces the level of fluctuations in the force response of *muscle gastrocnemius* in chronically alcoholized rats

Olexandr Motuziuk^{1,2} · Dmytro Nozdrenko¹ · Svitlana Prylutska³ · Kateryna Bogutska¹ · Iryna Mishchenko⁴ · Olga Abramchuk² · Sergii Khrapaty⁵ · Uwe Ritter⁶ · Yuriy Prylutsky¹

Received: 6 December 2022 / Accepted: 3 May 2023
© King Abdulaziz City for Science and Technology 2023

Abstract

The biomechanical parameters of *muscle gastrocnemius* contraction and biochemical parameters of blood and muscle tissue in rats after chronic alcoholization for 3, 6, and 9 months were studied. The oral administration of C₆₀ fullerene aqueous solution (C₆₀FAS) at doses of 0.5, 1, and 2 mg/kg throughout the experiment was used as a therapeutic agent. C₆₀FAS in each of the experimental groups was administered in three ways: 1 h before alcohol intake, together with alcohol, and 1 h after alcohol intake. The most significant positive effects were recorded when alcohol and C₆₀FAS were administered together at the optimal dose of 1 mg/kg. So, the increase in *muscle gastrocnemius* contraction force was $20 \pm 1\%$, $33 \pm 2\%$ and $65 \pm 3\%$ ($p < 0.05$) compared with control at 3, 6, and 9 months alcoholization, respectively, as well as a high level of its fluctuations correction was observed throughout the experiment. Biochemical parameters such as blood levels of creatinine, creatine phosphokinase, lactate and lactate dehydrogenase as well as pro- and antioxidant balance (content of hydrogen peroxide and reduced glutathione, as well as catalase, selenium-dependent glutathione peroxidase and superoxide dismutase activities) in *muscle gastrocnemius* tissues decreased from $15 \pm 2\%$ (3 months of alcoholization) to $45 \pm 2\%$ (9 months of alcoholization) ($p < 0.05$) compared to controls. The results indicate promising prospects for the use of water-soluble C₆₀ fullerenes, as powerful antioxidants, for the correction of pathological conditions of the muscular system arising from alcohol intoxication.

Keywords C₆₀ fullerene · *Muscle gastrocnemius* · Alcohol intoxication · Biomechanical parameters · Biochemical indicators

Introduction

Long-term alcohol consumption leads to the deterioration of skeletal muscle function, and muscle dysfunction develops regardless of age (Pendergast et al. 1993). The most common skeletal muscle myopathy is chronic alcoholic myopathy; more than 12% of people suffer from this condition for a long time (Estruch et al. 1993). The immediate consequence of alcoholic myopathy is a deterioration of muscle contractility and a reduction of muscle strength (Adachi et al. 2003a, b). It is caused both by the direct ultrastructural disintegration of myocytes and their atrophy (Vancampfort et al. 2020), and by changes in electrolytic homeostasis and bioelectrical activity (Estruch et al. 1993). A study (Nakahara et al. 2003) shows that alcoholic myopathy is more common than other diseases (e.g., cirrhosis, cardiomyopathy, or neuropathy) caused by alcoholism. This may be due to muscle protein degradation caused by the action of free radicals, which are generated in large quantities in alcoholic

✉ Yuriy Prylutsky
prylut@ukr.net

- ¹ ESC “Institute of Biology and Medicine”, Taras Shevchenko National University of Kyiv, Kiev 01601, Ukraine
- ² Faculty of Biology and Forestry, Lesya Ukrainka Volyn National University, Lutsk 43025, Ukraine
- ³ Faculty of Plant Protection, Biotechnology and Ecology, National University of Life and Environmental Science of Ukraine, Kiev 03041, Ukraine
- ⁴ Municipal Institution of Higher Education “Volyn Medical Institute” of the Volyn Region Council, Lutsk 43016, Ukraine
- ⁵ Department of Computational Mathematics and Computer Modeling, Interregional Academy of Personnel Management, Kiev 03039, Ukraine
- ⁶ Institute of Chemistry and Biotechnology, Technical University of Ilmenau, 98693 Ilmenau, Germany

tissues. Experiments on rats (Paice et al. 2003; Baumann et al. 2016) showed that prolonged alcohol intake inhibits muscle fiber protein type II synthesis, causes the dysfunction of neuromuscular excitation, reduces the number of muscle fibers and force response. The occurrence of muscle weakness in the development of alcoholic myopathy can be compensated to some extent by the increased activity of synergistic muscle groups, which leads to the imaginary feeling of no significant myotic damage (Julian et al. 2019). However, the manifestation of fluctuations during the phases of holding the maximal muscle strength efforts leads to the inability to make the correction of the precise joint positioning (Nozdrenko et al. 2005). As a result, the whole muscle, as a dynamic system, is unable to adequately implement the pools of neural activity coming from the central nervous system (Kostyukov et al. 2002, 2011). The nature and level of muscle dysfunctions are directly related to the level of development of pathological processes, the analysis of which can currently be performed exclusively at the phenomenological level.

The development of alcoholic myopathy primarily involves free radical formation in dysfunctional mitochondria (Adachi et al. 2003a, b; Pruznak et al. 2013), which leads to destruction of myocyte membrane structures and functional disorders of their enzyme systems (decreased Na^+/K^+ -ATPase activity and increased Ca^{2+} -ATPase activity). The inhibition of Na^+/K^+ -ATPase activity (Hong-Brown et al. 2021) suppresses the specific increase in Na^+ conduction that occurs in response to an adequate stimulus and thus prevents the development of excitation in myocytes. This can lead to a temporal imbalance in the timing of smooth tetanus establishment, which in turn increases the level of fluctuations in the force response of the muscle (the so-called 'tremor').

C_{60} fullerenes are known to be able to capture and inactivate free radicals efficiently in in vitro and in vivo systems (Eswaran 2018; Ferreira et al. 2018). Moreover, they penetrate into cells and localize preferentially in mitochondria (Grebinyk et al. 2018). In previous in vivo experiments, administration of biocompatible water-soluble C_{60} fullerenes (Halenova et al. 2020) has been shown to result in significant positive therapeutic effects following the ischemic injury initiation (Nozdrenko et al. 2021), fatigue (Prylutsky et al. 2017; Vereshchaka et al. 2018), atrophy (Nozdrenko et al. 2022a, b) and injury (Nozdrenko et al. 2022a, b) in skeletal muscles. However, the observed effects depend significantly on the doses used and the administration patterns of C_{60} fullerene aqueous solution (C_{60}FAS) against the background of the initiation of particular pathology.

Thus, the aim of the present study was to evaluate the effect of C_{60}FAS on the magnitude of the force response of *muscle gastrocnemius* in chronically alcoholized rats, in particular on its fluctuations depending on the dose and

administration scheme (prophylactic and therapeutic) of the drug.

Materials and methods

Preparation of C_{60}FAS

C_{60} fullerene (Sigma Cat. No. 379646) aqueous solution with purity of more than 99.96% has been prepared according to the protocol (Scharff et al. 2008; Ritter et al. 2015). The used method is based on transferring C_{60} molecules from organic solution into the aqueous phase by ultrasonic treatment. The prepared C_{60}FAS is a typical colloid fluid containing single C_{60} fullerenes (0.7 nm in diameter) as well as their nanoaggregates up to 100 nm (Ritter et al. 2015; Skamrova et al. 2014). The maximal concentration of C_{60} fullerenes in water obtained by this method was 0.15 mg/ml. The prepared C_{60}FAS is stable within 12–18 months at temperature + 4 °C.

The ethic statement

The study protocol was approved by the Bioethics Committee of the ESC "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv in accordance with the rules of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" and the norms of biomedical ethics in accordance with the Law of Ukraine No. 3447-IV 21.02.2006, Kyiv, "The Protection of Animals from Cruelty", as well as the European Union Directive (2010/63/EU) "On the protection of animals used for scientific purposes" during biomedical research.

Animal groups and model of animal alcoholization

The experiments were performed on male Wistar rats aged 1–10 months (at the end of the experiment).

The control group ($n = 10$) received 100% drinking water.

Animals in the "alcoholization" experimental groups ($n = 10$ in each group) were randomly selected. Each animal was placed in a separate cage to receive 40% ethanol in drinking water (Collins and Neafsey 2016), which meant that the experimental groups had no access to 100% water until the full dosed ethanol consumption (Song et al. 2002). Ethanol intake was calculated as 0.5% of animal's body weight. The ethanol dose was recalculated every 24 h during the whole experiment (D'Souza El-Guindy et al. 2010). Duration of alcoholization was 3, 6 and 9 months.

The experimental animals of "alcoholization + C_{60} " groups ($n = 10$ in each group) in addition to alcohol received C_{60}FAS at respective doses of 0.5, 1 and 2 mg/kg rat weight.

The C₆₀FAS dose was recalculated every 24 h throughout the experiment. The oral administration of C₆₀FAS in each of the "alcoholization + C₆₀" groups was performed in three ways: 1 h before alcohol intake, together with alcohol, and 1 h after alcohol intake. The experimental animals received free access to water only after the complete intake of drug.

It is important to note that the selected doses of C₆₀FAS in our experiments are significantly lower than the LD₅₀ value, which was 600 mg/kg body weight when administered orally to rats (Gharbi et al. 2005) and 721 mg/kg when administered intraperitoneally to mice (Prylutska et al. 2019).

Equipment for tensometry and surgical preparation of animals

A 12-bit analog-to-digital and digital-to-analog converter (ADC-DAC, Mouser Electronics, USA) was used to record the electrophysiological signals of muscle contraction (Nozdrenko et al. 2017). DAC output pulses were triggered by isolated stimulators (DS2A, Digitimer, Germany), which performed nerve stimulation. The input signals were fed through the amplifier (Mouser Electronics, USA) to the ADC and recorded with a sampling frequency of 10 kHz. A linear motor in the servo position was used to stretch the muscle and measure the force it developed. The efforts were measured using semiconductor strain gauges glued to rigid steel beams mounted on moving parts of the linear motor. The stiffness of the puller exceeded 0.06 N/mm, the time constant of transient processes did not exceed 60 ms.

The animals were anesthetized by intraperitoneal injection of nembutal (40 mg/kg). Experiment preparation included cannulation (*a. carotis communis sinistra*) for drug administration and pressure measurement, tracheotomy and laminectomy at the level of the lumbar spinal cord. The studied muscle *gastrocnemius* was freed from the surrounding tissues, the tendon part was cut in the distal part to the lower back. To prepare for the modulated stimulation of efferents in the corresponding segments, the ventral roots were cut directly at the places of their exit from the spinal cord. The filaments of the cut ventral roots were fixed on the stimulating electrodes and the stimulus sequence was cyclically distributed. Stimulation of efferents was carried out by electrical rectangular 2 ms pulses, generated by a pulse generator, with a frequency of 10–50 s⁻¹ and duration of 2, 4 and 6 s (Govbakh et al. 2021). The current strength, at which the muscle began to contract, was considered the threshold, and further stimulation was performed with a force of 1.3–1.4 threshold (Nozdrenko et al. 2017).

Biochemical analysis

The levels of enzymes (creatine phosphokinase and lactate dehydrogenase) as well as creatinine and lactate in the blood

plasma of the test animals and assessment of the level of oxidative processes in muscle tissues (content of hydrogen peroxide and reduced glutathione (GSH) as well as catalase (CAT), selenium-dependent glutathione peroxidase (GP_x) and superoxide dismutase (SOD) activities) as markers of muscle damage (Brancaccio et al. 2010) were determined using clinical diagnostic equipment [hemoanalyzers RNL-200 (Netherlands), ABX Micros ESV60 (France) and automatic analyzer Pentra C400 (France)].

Blood alcohol concentration (BAC) was determined at the end of the experiment by cardiac puncture using an AM1 alcohol analyzer (Analox Instruments Limited, UK).

Statistical analysis

Statistical processing of the measurement results was carried out by methods of variation statistics using both Origin 9.4 and Statistica 13.3 softwares. In the biomechanical studies, the fluctuations in the holding phase of the maximal force response of the muscle ($\Delta F = F_{\max} - F_{\min}$) as well as the amplitude of single muscle contractions before the transition of the force response to smooth tetanus ($f_{\min}$) were estimated. It must be emphasized, that each of the experimental curves of muscle contraction force is the result of averaging 10 similar measurements. In the biochemical studies, the biochemical indicators in blood plasma and muscle tissue were estimated. Five repeats were performed for each measurement. Data are expressed as the means $\pm$ SEM for each group. To determine the statistical significance of differences between the group means, a *t* test (for one group) or one-way ANOVA (for three groups) followed by a post hoc Dunnett multiple comparison test to compare all data vs control were used. A factorial ANOVA test was also used depending on such factors of variation as dose, treatment period and drug regimen. The underlying assumptions that the data follow Gaussian distributions and have identical standard deviations were tested using the methods of Shapiro-Wilk and Bartlett, respectively. The differences among experimental groups were judged to be statistically significant when $p < 0.05$.

Results and discussion

BAC value

At the end of the experiment, the BAC value in rats after chronic ethanol consumption ranged from 140 mg/dl (3 months of alcohol consumption) to 250 mg/dl (9 months of alcohol consumption). These data agree well with the results of a study (Song et al. 2002).

It is important to note that C₆₀FAS administration at a dose range of 0.5, 1 and 2 mg/kg in preventive (1 h before alcohol intake) and therapeutic (together with alcohol and

1 h after alcohol intake) regimens did not result in a significant change of BAC value throughout the experiment (data are not presented).

Muscle contraction force analysis

Figure 1 shows the mechanograms of the 10th muscle contraction in alcoholized rats at 50 s^{-1} for 2, 4 and 6 s stimulation. The recording of force responses to a 2 s stimulation signal revealed a sharp decrease in muscle contraction force by $19 \pm 1\%$, $22 \pm 2\%$ and $29 \pm 2\%$ from the control values at 3, 6, and 9 months of alcoholization, respectively. When force responses to the stimulation signal of 4 and 6 s were recorded, the decrease in muscle contraction force was $22 \pm 1\%$, $35 \pm 2\%$, $41 \pm 4\%$ and $25 \pm 1\%$, $39 \pm 2\%$, $79 \pm 4\%$ as compared to controls in the case of 3, 6, and 9 months of alcoholization, respectively. Rats receiving a mixture of alcohol and C_{60} FAS at a dose of 1 mg/kg throughout the period of alcoholization showed increases in muscle strength of $14 \pm 1\%$, $18 \pm 1\%$ and $35 \pm 2\%$ for 3 months of alcoholization, $19 \pm 1\%$, $28 \pm 2\%$ and $44 \pm 2\%$ at 6-month alcoholization and finally by $23 \pm 1\%$, $36 \pm 2\%$ and $59 \pm 2\%$ at 9-month alcoholization when stimulation of 2, 4 and 6 s, respectively, was applied relative to the “alcoholization” group (Fig. 1).

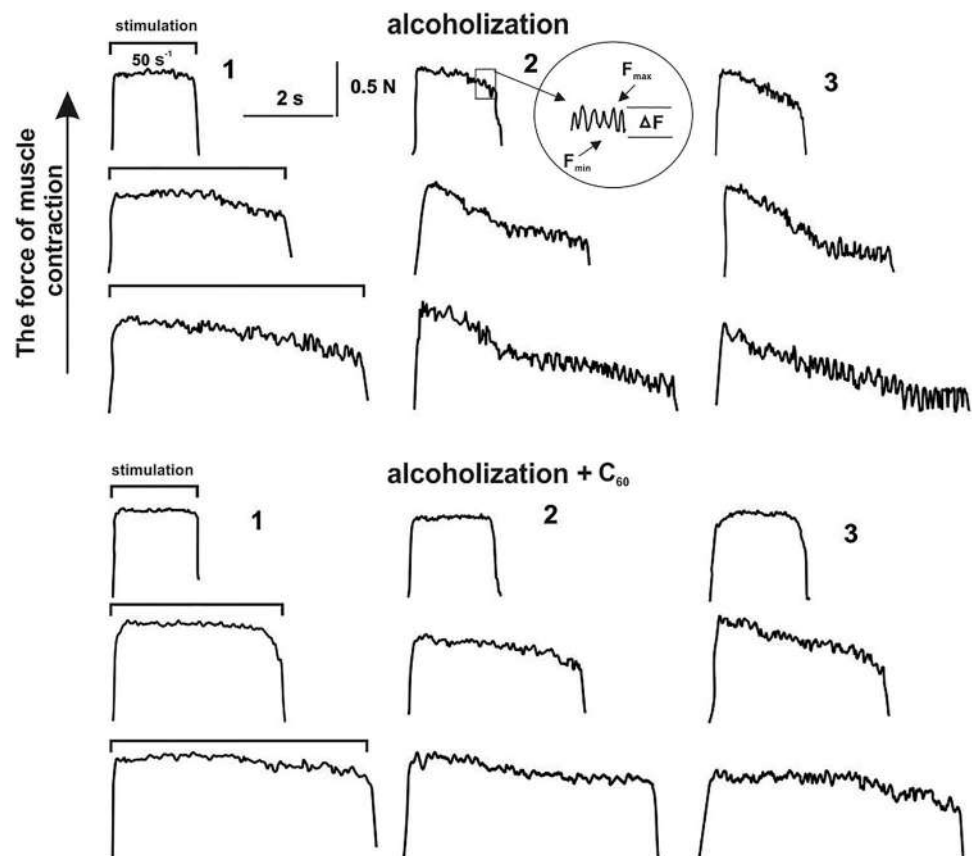
Alcoholization for 3 months increased fluctuations in the holding phase of the maximal force response of the muscle ($\Delta F = F_{\max} - F_{\min}$) from 0.03 mN at 2 s stimulation to 0.06 mN at 6 s one. There was a sharp increase in this index with increasing duration of alcoholization. So, at 9 months of alcohol intake the level of fluctuations was more than 1.2 mN (Figs. 1, 2). In animals receiving a mixture of alcohol and C_{60} FAS at a dose of 1 mg/kg throughout the period of alcoholization, there was a $15 \pm 1\%$ decrease in the level of muscle fluctuations at 3 months alcoholization, $32 \pm 2\%$ at 6 months and $70 \pm 3\%$ at 9 months relative to the “alcoholization” group (Figs. 1, 2).

Reducing the dose of C_{60} FAS to 0.5 mg/kg during the studied periods of alcoholization in rats showed the significant worsening of the effects described above (Fig. 2). Increasing the dose of C_{60} FAS to 2 mg/kg only slightly increased the positive effect recorded with C_{60} FAS at a dose of 1 mg/kg, namely by $7 \pm 1\%$, $6 \pm 1\%$ and $8 \pm 1\%$ in cases 3, 6 and 9 months of alcoholization, respectively. Thus, it can be assumed that the dose of C_{60} FAS 1 mg/kg is optimal.

An analysis of the methods of introducing C_{60} FAS during alcohol intoxication of animals revealed its most significant effect when C_{60} FAS was mixed with alcohol (Fig. 2).

Thus, when using C_{60} FAS (1 mg/kg) 1 h before alcohol intake (prophylactic regimen), its effectiveness was lower

Fig. 1 The strength of the muscle *gastrocnemius* 10th contraction of alcoholized rats caused by 2, 4 and 6 s non-relaxation stimulation pools: 1, 2 and 3—alcoholized rats for 3, 6 and 9 months, respectively; alcoholization + C_{60} (1 mg/kg)—rats receiving a mixture of alcohol and C_{60} FAS at a dose of 1 mg/kg during the entire period of alcoholization; $F_{\max}$ and $F_{\min}$ —maximum and minimum levels of fluctuation amplitudes of the force response at the phases of holding the maximum force of muscle contraction



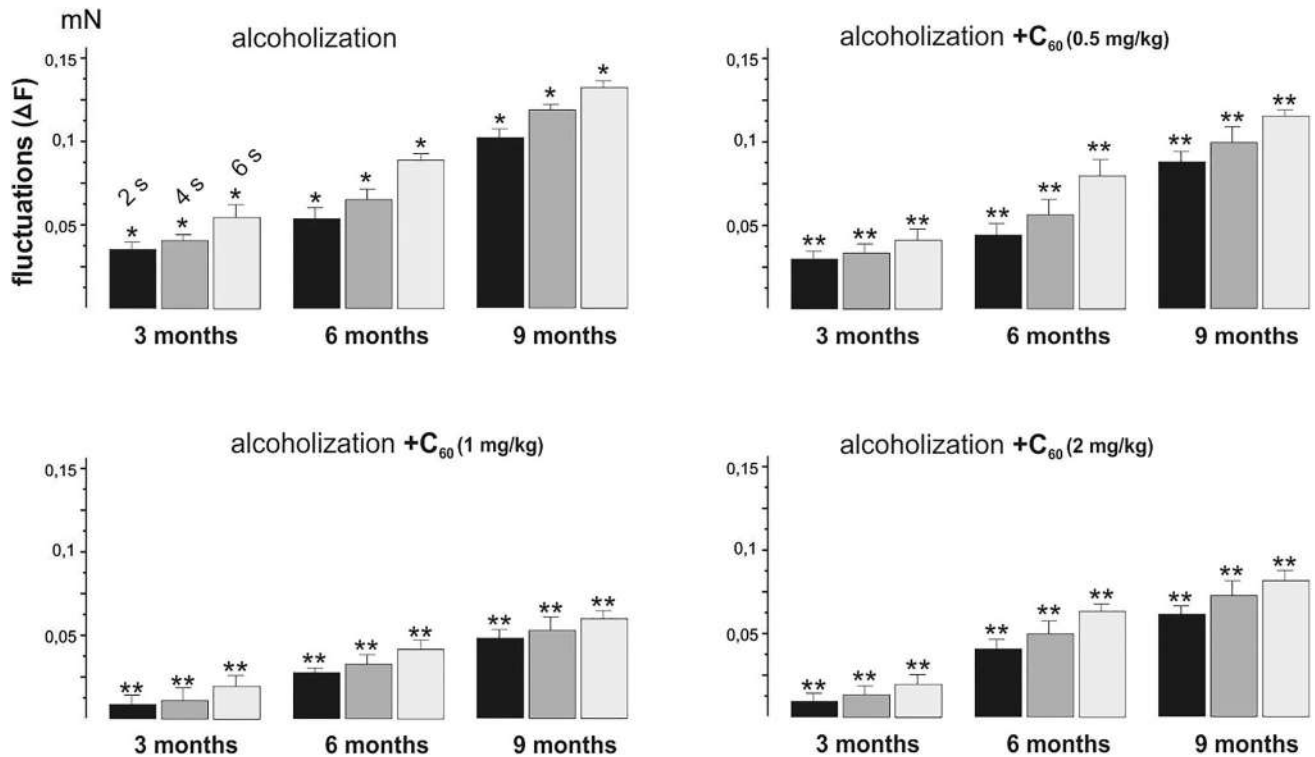


Fig. 2 Fluctuations levels ($\Delta F = F_{\max} - F_{\min}$) in the *muscle gastrocnemius* contraction of alcoholized rats induced by 2, 4 and 6 s non-relaxation stimulation pools: alcoholization + C_{60} (0.5, 1 and 2 mg/kg)—rats treated with alcohol mixture and C_{60} FAS at doses

0.5, 1 and 2 mg/kg for the whole period of alcoholization; 3, 6 and 9 months—alcoholization lasting 3, 6 and 9 months, respectively. * $p < 0.05$; ** $p < 0.05$ relative to the "alcoholization" group

by $10 \pm 1\%$, $13 \pm 1\%$ and $17 \pm 1\%$ at 3, 6 and 9 months of alcoholization, respectively, compared with the use of alcohol and C_{60} FAS together (Figs. 2, 3). When taking C_{60} FAS (1 mg/kg) 1 h after alcohol intake (therapeutic regimen), its effectiveness was lower by $9 \pm 1\%$, $16 \pm 1\%$ and $30 \pm 2\%$ at 3, 6 and 9 months of alcoholization,

respectively, compared with the use of alcohol and C_{60} FAS together (Figs. 2, 3).

The increased level of fluctuational amplitudes of the force response in alcoholic animals may be associated with the impaired processes of a single contractions summation with subsequent temporal delays preventing the formation

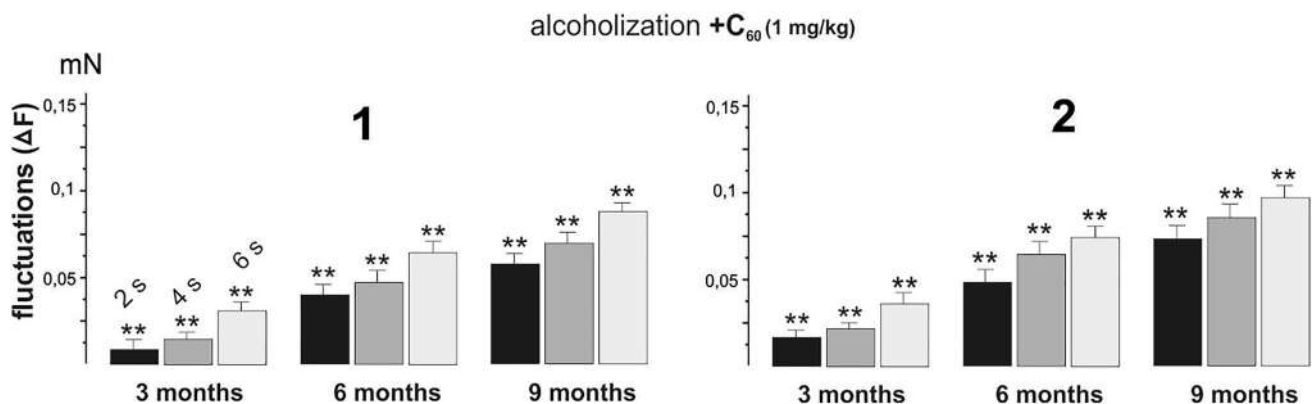


Fig. 3 Fluctuations levels ($\Delta F = F_{\max} - F_{\min}$) during *muscle gastrocnemius* contraction in alcoholic rats induced by 2, 4 and 6 s non-relaxation stimulation pools: alcoholization + C_{60} (1 mg/kg)—rats receiving alcohol and C_{60} FAS at a dose of 1 mg/kg throughout the

entire period of alcoholization; 3, 6 and 9 months—alcoholization lasting 3, 6 and 9 months, respectively; 1 and 2—administration of C_{60} FAS 1 h before alcohol intake and 1 h after alcohol intake, respectively. ** $p < 0.05$ relative to the "alcoholization" group

of sustained maximum muscle contraction (Khoma et al. 2014). Therefore, we analyzed the biomechanical parameters of *muscle gastrocnemius* transition from serrated to smooth tetanus (Figs. 4, 5) using a therapeutic regimen of alcohol administration together with C₆₀FAS at an optimal dose of 1 mg/kg.

Figure 4 shows the mechanograms of *muscle gastrocnemius* contraction with modulated stimulation at frequencies of 10, 15, and 20 s⁻¹. The single contractions amplitude of the muscle of alcoholized rats during stimulation with a frequency of 10 s⁻¹ increased compared to control values by 15 ± 1%, 24 ± 1% and 56 ± 2% with alcoholization lasting 3, 6 and 9 months, respectively. When stimulated with frequencies of 15 and 20 s⁻¹, these indicators increased by 19 ± 1%, 26 ± 1%, 70 ± 2% and 27 ± 1%, 48 ± 2%, 80 ± 3%, respectively. It is important to emphasize that in the considered cases, the *muscle gastrocnemius* of the alcoholized rats did not switch into the state of smooth tetanus.

C₆₀FAS administration has stabilized the mechanograms, bringing them closer to control values in the case of 3-month alcoholization of rats. With the increase in the duration of alcoholization in rats up to 6 and 9 months, the positive effect of C₆₀FAS administration was 20 ± 1%, 25 ± 2%, 30 ± 2% and 24 ± 1%, 37 ± 2%, 53 ± 3%, respectively, at stimulation at frequencies of 10, 15 and 20 s⁻¹ (Fig. 4).

The amplitude of single muscle contractions before the transition of the force response to smooth tetanus ($f_{\min}$) (Fig. 5) was significantly increased by C₆₀FAS

administration, namely by 29 ± 1%, 33 ± 1% and 40 ± 2% at 3, 6 and 9 months, respectively, contributing to reduced fluctuations in *muscle gastrocnemius* contractions during the maximum force response retention phases.

The analysis of biochemical parameters in blood plasma and muscle tissue

The analysis of blood plasma biochemical parameters, which are responsible for skeletal muscle function quality and used to diagnose the development of muscular myopathies (Gibala et al. 1995), in particular changes in creatinine, creatine phosphokinase, lactate and lactate dehydrogenase levels, made it possible to assess physiological changes in a skeletal muscle function caused by long-term alcoholization of animals. All these biochemical markers were found to have a pronounced upward trend. Changes in creatinine, a product formed in muscle when intramuscular structures are destroyed, allowed to estimate the level of myocyte damage. When alcohol and C₆₀FAS at a dose of 1 mg/kg were coadministered, creatinine levels decreased by 17 ± 1%, 19 ± 1% and 36 ± 2% with alcoholization lasting 3, 6 and 9 months, respectively (Fig. 6).

It is important to emphasise that C₆₀FAS administration 1 h before and 1 h after alcohol ingestion decreased creatinine levels only slightly, by 9 ± 1% and 11 ± 1% on average, respectively.

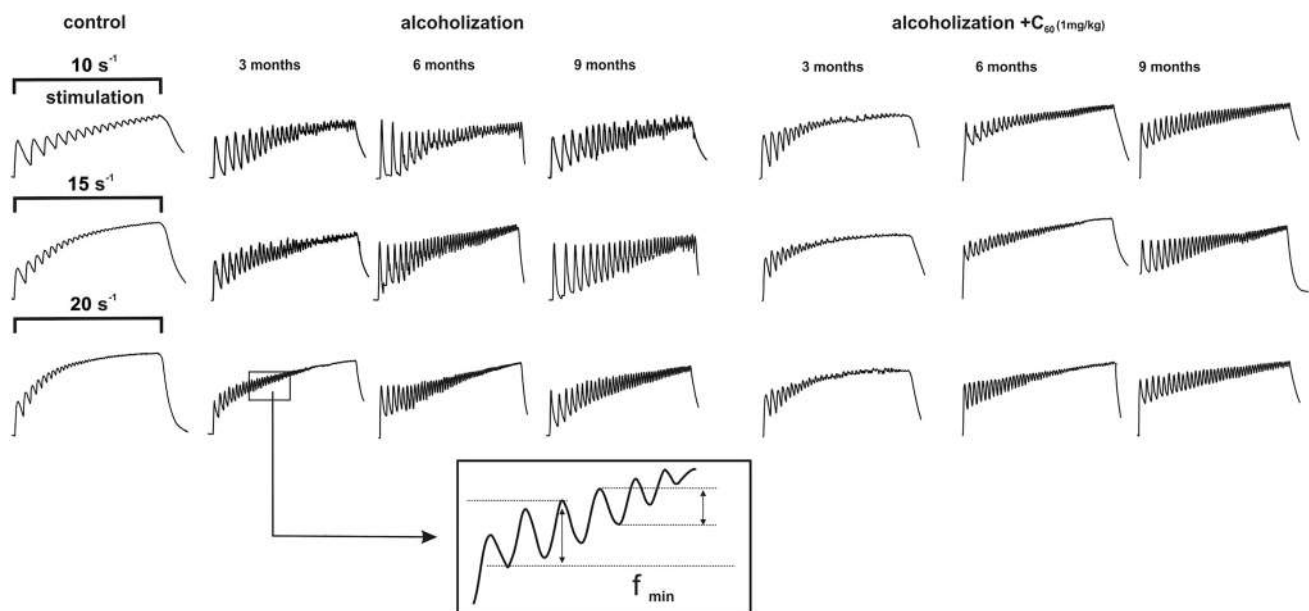


Fig. 4 Biomechanical parameters of *muscle gastrocnemius* transition from serrated to smooth tetanus when applying a modulated stimulation with increasing frequencies of 10, 15 and 20 s⁻¹ for 6 s: control—rats receiving 100% drinking water; alcoholization—alcoholized rats; 3, 6 and 9 months—alcoholization lasting 3, 6 and

9 months, respectively; alcoholization + C₆₀ (1 mg/kg)—rats receiving a mixture of alcohol and C₆₀FAS at a dose of 1 mg/kg for the duration of alcoholization; $f_{\min}$ —minimum force response value in one serrated tetanus (reducing this parameter to zero value results in smooth tetanus)

Fig. 5 Change in $f_{\min}$ parameter for each of the single contractions before the transition of the force response of *muscle gastrocnemius* into smooth tetanus when applying a stimulation signal with frequency of 20 s^{-1} : control—rats receiving 100% drinking water; alcoholization—alcoholized rats; 3, 6 and 9 months—alcoholization for 3, 6 and 9 months, respectively; alcoholization + C_{60} (1 mg/kg)—rats receiving mixture of alcohol and C_{60} FAS in a dose of 1 mg/kg for the whole period of alcoholization. # $p < 0.05$; * $p < 0.05$ relative to the control group; ** $p < 0.05$ relative to the “alcoholization” group

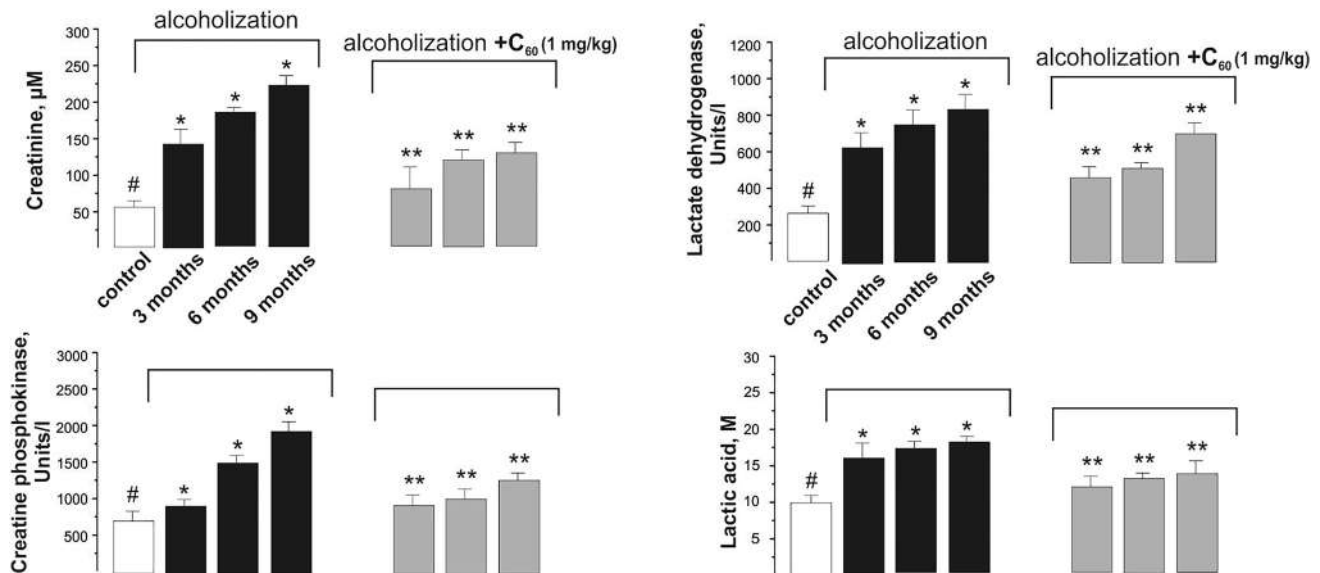
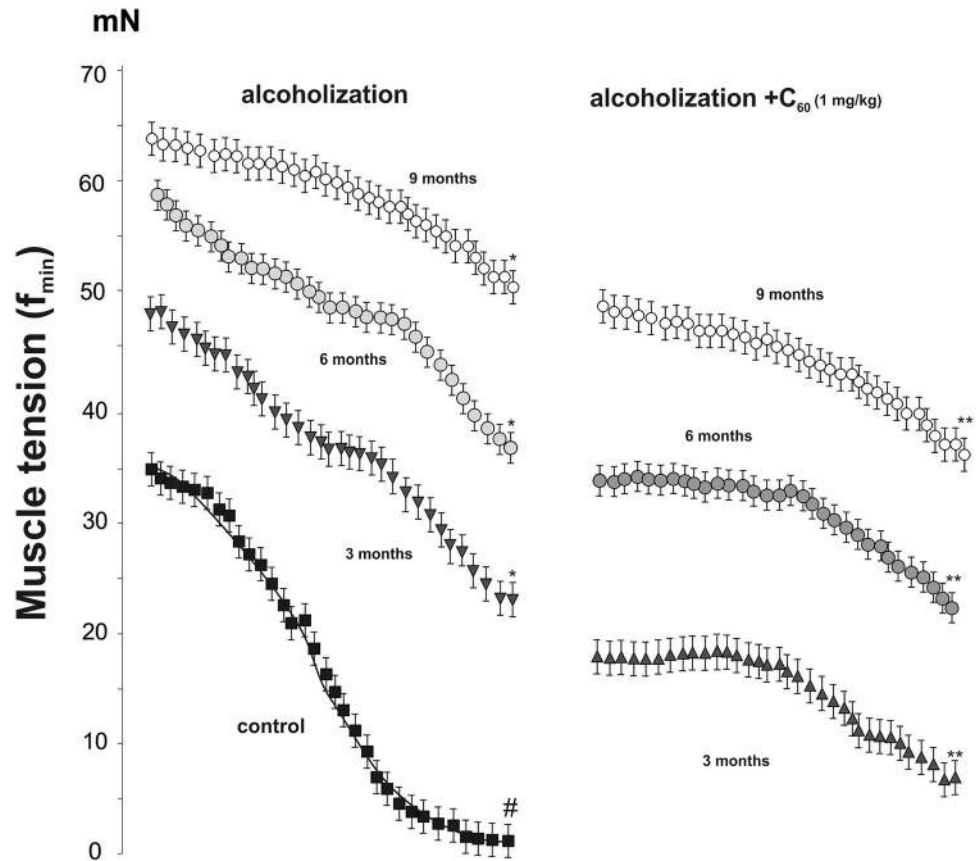


Fig. 6 Changes in creatinine, creatine phosphokinase, lactate dehydrogenase and lactate levels in blood plasma of alcoholized rats after the application of a relaxation-free stimulation with 30 pools of 50 s^{-1} for 6 s: control—rats receiving 100% drinking water; alcoholization—alcoholized rats; alcoholization + C_{60} (1 mg/kg)—rats receive-

ing a mixture of alcohol and C_{60} FAS at a dose of 1 mg/kg for the duration of alcoholization; 3, 6 and 9 months—alcoholization lasting 3, 6 and 9 months, respectively. # $p < 0.05$; * $p < 0.05$ relative to the control group; ** $p < 0.05$ relative to the “alcoholization” group

One of the important markers of pathological processes in a muscle is a change in the creatine phosphokinase concentration, an enzyme from the energy supply system of skeletal muscle cells. When muscles are damaged during active functioning, this enzyme is released from the cells and there is a corresponding increase in its quantity in the blood. The concomitant administration of alcohol and C₆₀FAS at a dose of 1 mg/kg decreased creatine phosphokinase levels by $13 \pm 1\%$, $19 \pm 1\%$ and $28 \pm 2\%$ with alcoholization lasting 3, 6 and 9 months, respectively (Fig. 6).

During the development of pathological reactions cascade after the muscle myopathies, a significant depletion of cellular energetic substances occurs, leading to a disturbance of homeostasis and a loss of ionic gradient due to cell membranes. This leads to the accumulation of lactate and H⁺ ions and, consequently, to an acidification of the pH of the intra- and extracellular media. Coadministration of alcohol and C₆₀FAS at a dose of 1 mg/kg decreased lactate levels by $19 \pm 1\%$, $28 \pm 2\%$ and $37 \pm 2\%$ with alcoholization lasting 3, 6 and 9 months, respectively (Fig. 6).

The level of changes in lactate dehydrogenase, an enzyme that catalyzes lactic acid oxidation, allowed the overall performance status of injured muscle to be assessed after prolonged activation. When alcohol and C₆₀FAS at a dose of 1 mg/kg were coadministered, lactate dehydrogenase levels

decreased by $15 \pm 1\%$, $29 \pm 2\%$ and $40 \pm 2\%$ during alcoholization for 3, 6 and 9 months, respectively (Fig. 6).

In the development of muscle pathology, changes in muscle antioxidant content are an important criterion determining the level of physiological disorders of muscle cells. The explanation for the obtained positive therapeutic effect of water-soluble C₆₀ fullerenes may be their powerful antioxidant properties (Eswaran 2018; Ferreira et al. 2018; Gharbi et al. 2005). The evaluation of the levels of pro- and antioxidant balance in the tissues of the muscle under study is essential to confirm this assumption.

Figure 7 shows the results of biochemical tests indicating the increased levels of peroxidation and oxidative stress after the alcoholized muscle stimulation. So, the H₂O₂ concentration increased with the increasing duration of alcoholization and was $151 \pm 5\%$, $327 \pm 6\%$ and $414 \pm 6\%$ when alcoholization lasted 3, 6 and 9 months, respectively, compared with the control. Coadministration of alcohol and C₆₀FAS at a dose of 1 mg/kg resulted in a $28 \pm 2\%$, $44 \pm 2\%$ and $50 \pm 2\%$ reduction in H₂O₂ in muscle *gastrocnemius*, respectively. The observed inhibition of excessive accumulation of oxygen derivative H₂O₂ in muscle may be mediated by the antiradical properties of C₆₀ fullerene. The results confirm that C₆₀ fullerenes are able to penetrate through plasma membranes and

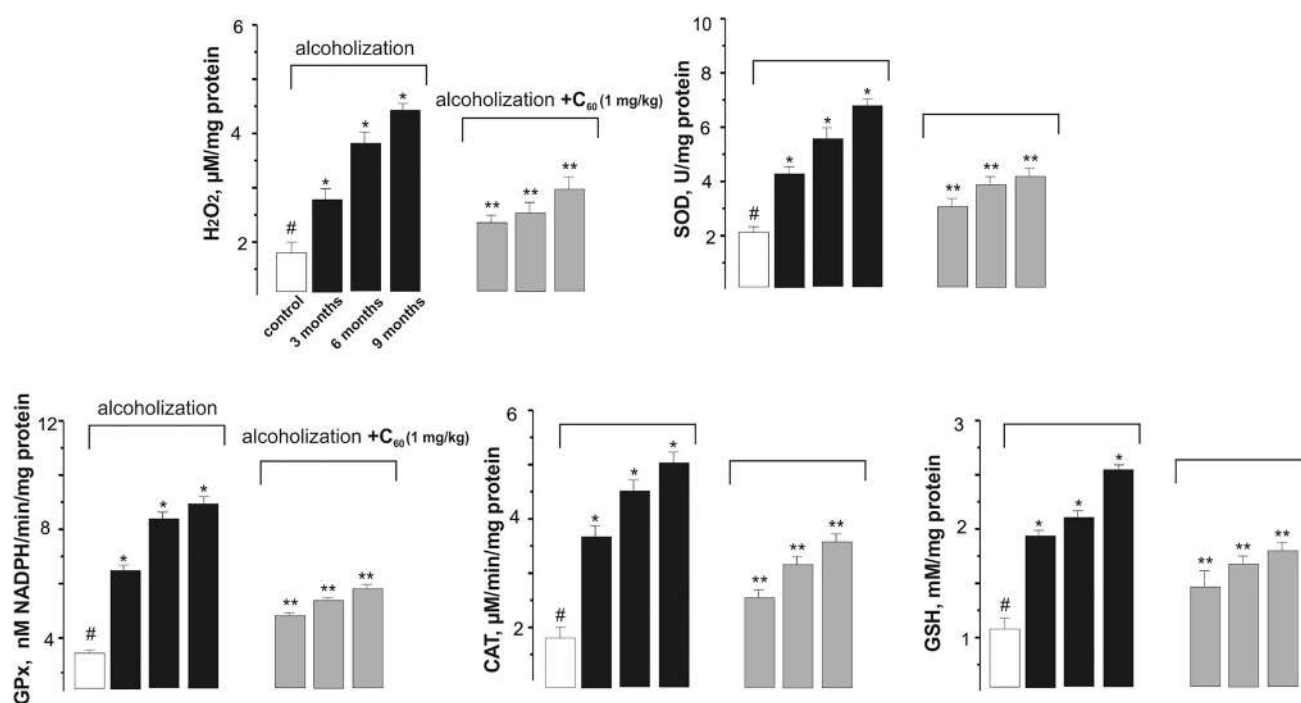


Fig. 7 Indicators of pro- and antioxidant balance (H₂O₂, SOD, GPx, CAT and GSH) in rat muscle *gastrocnemius* tissue after the relaxation-free stimulation with 50 pools of 20 s^{-1} for 6 s: control—rats receiving 100% drinking water; alcoholization—alcoholized rats; alcoholization + C₆₀ (1 mg/kg)—rats receiving a mixture of alcohol

and C₆₀FAS at a dose of 1 mg/kg for the duration of alcoholization; 3, 6 and 9 months—alcoholization lasting 3, 6 and 9 months, respectively. # $p < 0.05$; * $p < 0.05$ relative to the control group; ** $p < 0.05$ relative to the "alcoholization" group

accumulate in selected tissues, including muscle, without evidence of damage (Prylutska et al. 2019; Prylutsky et al. 2016).

Antioxidant defense enzymes such as SOD, CAT and GP_x play a key role in the regulation mechanisms of free-radical and peroxide processes. The most powerful natural antioxidant and the enzyme of the first link of antioxidant protection is SOD, which carries out the reaction of superoxide anion-radical dismutation and converts them into less reactive hydrogen peroxide molecules (Sen 2001). Studies (Hollander et al. 1999; Suzuki et al. 2000) showed that SOD activity in rat skeletal muscles increased during exercise or the development of pathological processes, predominantly in oxidative muscles with a high content of type I and IIa fibers. In our experiment with the electrical stimulation of muscles against the background of coadministration of alcohol and C₆₀FAS at a dose of 1 mg/kg, a gradual decrease of SOD activity in *muscle gastrocnemius* was found, namely, by $20 \pm 1\%$, $29 \pm 1\%$ and $43 \pm 2\%$ after 3, 6 and 9 months of alcoholization of rats, respectively.

The change in CAT activity in *muscle gastrocnemius* when alcohol and C₆₀FAS at a dose of 1 mg/kg were coadministered was $12 \pm 1\%$, $34 \pm 2\%$ and $47 \pm 2\%$ after 3, 6 and 9 months of alcoholization in rats, respectively.

Cellular mechanisms of antioxidant protection are also associated with the functioning of powerful glutathione link (Sen 2001). Along with antiradical enzymes, the glutathione system is one of the active components of the body's antioxidant protection, which plays a significant role in attenuating the pathological process in the development of muscle myopathy, as it not only prevents the course of free-radical reactions, but also provides effective elimination of the final metabolites of lipid peroxidation (Ji 2008). In our study, after coadministration of alcohol and C₆₀FAS at a dose of 1 mg/kg, GP_x activity in *muscle gastrocnemius* decreased by $17 \pm 1\%$, $23 \pm 1\%$ and $40 \pm 2\%$ after 3, 6 and 9 months of alcohol exposure in rats, respectively.

The combined administration of alcohol and C₆₀FAS at a dose of 1 mg/kg resulted in $13 \pm 1\%$, $27 \pm 1\%$ and $40 \pm 2\%$ change in GSH content in *muscle gastrocnemius* after 3, 6 and 9 months of alcohol exposure in rats, respectively. Consequently, the administration of C₆₀FAS led to an increase in the efficiency of the antioxidant defense system by increasing GSH content in muscle, thereby increasing muscle tolerance to physical exertion. This is in good agreement with the results (Gonchar et al. 2018), where it was shown that C₆₀ fullerene is able to influence the processes of glutathione synthesis and metabolism in various tissues, including muscle, under various pathological conditions by modulating the Nrf2/ARE-antioxidant pathway.

Thus, in all conducted tests there is a positive change in the studied biochemical parameters by about $(15-30) \pm 2\%$ when C₆₀FAS at a dose of 1 mg/kg is administered together

with alcohol. It is also worth noticing that C₆₀FAS is $(7-10) \pm 2\%$ effective with other methods of administration.

On the basis of the obtained data it can be concluded that the application of water-soluble C₆₀ fullerenes contributes to the reduction of oxidative processes in muscles by maintaining the balance between pro-oxidants and antioxidant protection system, which prevents the negative effect of reactive oxygen species on cellular and subcellular structures in the development of alcoholic myopathy in rats. This indicates a compensatory activation of the endogenous antioxidant system by C₆₀ fullerene during pathological changes in the rat *muscle gastrocnemius* caused by chronic alcohol consumption. In our opinion, C₆₀ fullerene affects the activity of endogenous antioxidants, suppressing the occurrence of destruction in muscle and thus reducing its degradation.

Conclusion

Thus, for the first time in the model of chronically alcoholized rats it was shown that combined administration of ethanol and C₆₀FAS at the optimal dose of 1 mg/kg shows a higher level of muscle force response correction compared to other methods of this drug administration, in particular it reduces the level of fluctuations of force response during the phases of maximum force effort (the so-called "tremor"), which was confirmed by the biochemical tests. It can be assumed, that a mixture of alcohol and C₆₀FAS in alcoholic beverages, without changing their taste, can bring significant positive effect on myopathic dysfunctions caused by alcohol intoxication. Summarizing, this finding opens the possibility of using water-soluble C₆₀ fullerenes as promising pharmacological nanoagents capable of correcting the pathological conditions of the muscular system arising in the development of alcoholic myopathy.

Author contributions OM, DN and KB: biomechanical analysis. SP, IM and OA: biochemical analysis. SK and UR: preparation and characterization of the samples. YP: coordination of the research work, analysis of the data and preparing the manuscript. All authors have read and agreed to the published version of the manuscript.

Data availability All data and materials are available.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

- Adachi J, Asanoa M, Ueno Y, Niemelä O, Ohlendieck K, Peters TJ, Preedy VR (2003a) Alcoholic muscle disease and biomembrane

- perturbations (review). *J Nutr Biochem* 14:616–625. [https://doi.org/10.1016/s0955-2863\(03\)00114-1](https://doi.org/10.1016/s0955-2863(03)00114-1)
- Adachi J, Fujita T, Kudo R, Asano M, Nurhantari Y, Ueno Y (2003b) 7-hydroperoxycholesterol and oxysterols as indices of oxidative stress: chronic ethanol feeding and rat skeletal muscle. *Legal Med* 5:105–109. [https://doi.org/10.1016/s1344-6223\(02\)00077-9](https://doi.org/10.1016/s1344-6223(02)00077-9)
- Baumann CW, Kwak D, Liu HM, Thompson LV (2016) Age-induced oxidative stress: how does it influence skeletal muscle quantity and quality? *J Appl Physiol* 121:1047–1052. <https://doi.org/10.1152/japplphysiol.00321.2016>
- Brancaccio P, Lippi G, Maffulli N (2010) Biochemical markers of muscular damage. *Clin Chem Lab Med* 48:757–767. <https://doi.org/10.1515/CCLM.2010.179>
- Collins MA, Neafsey EJ (2016) Alcohol, excitotoxicity and adult brain damage: an experimentally unproven chain-of-events. *Front Mol Neurosci* 9:8. <https://doi.org/10.3389/fnmol.2016.00008>
- D'Souza El-Guindy NB, Kovacs EJ, De Witte P, Spies C, Littleton JM, De Villiers WJS, Lott AJ, Plackett TP, Lanzke N, Meadows GG (2010) Laboratory models available to study alcohol-induced organ damage and immune variations: choosing the appropriate model. *Alcohol Clin Exp Res* 34:1489–1511. <https://doi.org/10.1111/j.1530-0277.2010.01234.x>
- Estruch R, Nicolas JM, Villegas E, Junqué A, Urbano-Márquez A (1993) Relationship between ethanol related diseases and nutritional status in chronically alcoholic men. *Alcohol* 28:543–550
- Eswaran SV (2018) Water soluble nanocarbon materials: a panacea for all? *Curr Sci* 114:1846–1850. <https://doi.org/10.18520/cs/v114/109/1846-1850>
- Ferreira CA, Ni D, Rosenkrans ZT, Cai W (2018) Scavenging of reactive oxygen and nitrogen species with nanomaterials. *Nano Res* 11:4955–4984. <https://doi.org/10.1007/s12274-018-2092-y>
- Gharbi N, Pressac M, Hadchouel M, Szwarc H, Wilson SR, Moussa F (2005) [60]fullerene is a powerful antioxidant in vivo with no acute or subacute toxicity. *Nano Lett* 5:2578–2585. <https://doi.org/10.1021/nl051866b>
- Gibala MJ, MacDougall JD, Tarnopolsky MA, Stauber WT, Elorriaga A (1995) Changes in human skeletal muscle ultrastructure and force production after acute resistance exercise. *J Appl Physiol* 78:702–708. <https://doi.org/10.1152/jappl.1995.78.2.702>
- Gonchar O, Maznychenko A, Bulgakova N, Vereschaka I, Tomiak T, Ritter U, Prylutsky Y, Mankovska I, Kostyukov A (2018) C₆₀ Fullerene prevents restraint stress-induced oxidative disorders in rat tissues: possible involvement of the Nrf2/ARE-antioxidant pathway. *Oxidative Med Cell Longevity*. <https://doi.org/10.1155/2018/2518676>
- Govbakh I, Kyryk V, Ustyomenko A, Rubtsov V, Tsupykov O, Bulgakova N, Zavadovskiy D, Sokolowska I, Maznychenko A (2021) Stem cell therapy enhances motor activity of triceps *surae* muscle in mice with hereditary peripheral neuropathy. *Int J Mol Sci* 22:12026. <https://doi.org/10.3390/ijms222112026>
- Grebinyk A, Grebinyk S, Prylutska S, Ritter U, Matyshevska O, Dandekar T, Frohme M (2018) C₆₀ fullerene accumulation in human leukemic cells and perspectives of LED-mediated photodynamic therapy. *Free Radic Biol Med* 124:319–327. <https://doi.org/10.1016/j.freeradbiomed.2018.06.022>
- Halenova T, Raksha N, Savchuk O, Ostapchenko L, Prylutsky Y, Ritter U, Scharff P (2020) Evaluation of the biocompatibility of water-soluble pristine C₆₀ fullerenes in rabbit. *BioNanoSci* 10:721–730. <https://doi.org/10.1007/s12668-020-00762-w>
- Hollander J, Fiebig R, Gore M, Bejma J, Ookawara T, Ohno H, Ji LL (1999) Superoxide dismutase gene expression in skeletal muscle: fiber-specific adaptation to endurance training. *Am J Physiol* 277:R856–R862. <https://doi.org/10.1152/ajpregu.1999.277.3.R856>
- Hong-Brown LQ, Frost RA, Lang CH (2021) Alcohol impairs protein synthesis and degradation in cultured skeletal muscle cells. *Alcohol Clin Exp Res* 25:1373–1382
- Ji LL (2008) Modulation of skeletal muscle antioxidant defense by exercise: Role of redox signaling. *Free Radic Biol Med* 44:142–152. <https://doi.org/10.1016/j.freeradbiomed.2007.02.031>
- Julian T, Glasgow N, Syeed R, Zis P (2019) Alcohol-related peripheral neuropathy: a systematic review and meta-analysis. *J Neurol* 266:2907–2919. <https://doi.org/10.1007/s00415-018-9123-1>
- Khoma OM, Zavadovs'kyi DA, Nozdrenko DN, Dolhopolov OV, Miroshnychenko MS, Motuziuk OP (2014) Dynamics of ischemic skeletal soleus muscle contraction in rats. *Fiziol Zh* 60:34–40
- Kostyukov AI, Kalezić I, Serenko SG, Ljubicavljević M, Windhorst U, Johansson H (2002) Spreading of fatigue-related effects from active to inactive parts in the medial gastrocnemius muscle of the cat. *Eur J Appl Physiol* 86:295–307. <https://doi.org/10.1007/s00421-001-0550-8>
- Kostyukov AI, Lytvynenko SV, Bulgakova NV, Gorkovenko AV (2011) Changes in the threshold of generation of action potentials by spinal motoneurons under conditions of their natural activation. *Neurophysiol* 43:182–191. <https://doi.org/10.1007/s11062-011-9201-9>
- Nakahara T, Hashimoto K, Hirano M, Koll M, Martin CR, Preedy VR (2003) Acute and chronic effects of alcohol exposure on skeletal muscle c-myc, p53, and Bcl-2 mRNA expression. *Am J Physiol Endocrinol Metab* 285:E1273–E1281. <https://doi.org/10.1152/ajpendo.00019.2003>
- Nozdrenko DN, Shut AN, Prylutsky YuI (2005) The possible molecular mechanism of the nonlinearity muscle contraction and its experimental substantiation. *Biopolym Cell* 21:80–83. <https://doi.org/10.7124/bc.0006E0>
- Nozdrenko DM, Zavadovsky DO, Matvienko TY, Zay SY, Bogutska KI, Prylutsky YI, Ritter U, Scharff P (2017) C₆₀ fullerene as promising therapeutic agent for the prevention and correction of functioning skeletal muscle at ischemic injury. *Nanoscale Res Lett* 12:115. <https://doi.org/10.1186/s11671-017-1876-4>
- Nozdrenko D, Matvienko T, Vygovska O, Bogutska K, Motuziuk O, Nurishchenko N, Prylutsky Y, Scharff P, Ritter U (2021) Protective effect of water-soluble C₆₀ fullerene nanoparticles on the ischemia-reperfusion injury of the muscle soleus in rats. *Int J Mol Sci* 22:6812. <https://doi.org/10.3390/ijms22136812>
- Nozdrenko D, Matvienko T, Vygovska O, Soroca V, Bogutska K, Zholos A, Scharff P, Ritter U, Prylutsky Y (2022a) Post-traumatic recovery of muscle soleus in rats is improved via synergistic effect of C₆₀ fullerene and TRPM8 agonist menthol. *Appl Nanosci* 12:467–478. <https://doi.org/10.1007/s13204-021-01703-z>
- Nozdrenko D, Prylutska S, Bogutska K, Nurishchenko NY, Abramchuk O, Motuziuk O, Prylutsky Y, Scharff P, Ritter U (2022b) Effect of C₆₀ fullerene on recovery of muscle soleus in rats after atrophy induced by achillectomy. *Life* 12:332. <https://doi.org/10.3390/life12030332>
- Paice AG, Hesketh JE, Towner P, Hirako M, Peters TJ, Preedy VR (2003) No change in apoptosis in skeletal muscle exposed acutely or chronically to alcohol. *Addict Biol* 8:97–105. <https://doi.org/10.1080/1355621031000069945>
- Pendergast DR, York JL, Fisher NM (1993) A survey of muscle function in detoxified alcoholics. *Alcohol* 7:361–366. [https://doi.org/10.1016/0741-8329\(90\)90096-u](https://doi.org/10.1016/0741-8329(90)90096-u)
- Pruznak AM, Nystrom J, Lang CH (2013) Direct central nervous system effect of alcohol alters synthesis and degradation of skeletal muscle protein. *Alcohol Alcohol* 48:138–145. <https://doi.org/10.1093/alcal/ags113>
- Prylutska SV, Grebinyk AG, Lynchak OV, Byelinska IV, Cherepanov VV, Tauscher E, Matyshevska OP, Prylutsky YuI, Rybalchenko VK, Ritter U, Frohme M (2019) In vitro and in vivo toxicity of pristine C₆₀ fullerene aqueous colloid solution. *Fullerenes*

- Nanotubes Carbon Nanostruct 27(715):728. <https://doi.org/10.1080/1536383X.2019.1634055>
- Prylutsky Yu, Bychko A, Sokolova V, Prylutska S, Evstigneev M, Rybalchenko V, Epple M, Scharff P (2016) Interaction of C_{60} fullerene complexed to doxorubicin with model bilipid membranes and its uptake by HeLa cells. *Mater Sci Eng C* 59:398–403
- Prylutsky YuI, Vereshchaka IV, Maznychenko AV, Bulgakova NV, Gonchar OO, Kyzyma OA, Ritter U, Scharff P, Tomiak T, Nozdrenko DM, Mischenko IV, Kostyukov AI (2017) C_{60} fullerene as promising therapeutic agent for correcting and preventing skeletal muscle fatigue. *J Nanobiotechnol* 15:8. <https://doi.org/10.1186/s12951-016-0246-1>
- Ritter U, Prylutsky YuI, Evstigneev MP, Davidenko NA, Cherepanov VV, Senenko AI, Marchenko OA, Naumovets AG (2015) Structural features of highly stable reproducible C_{60} fullerene aqueous colloid solution probed by various techniques. *Fullerenes Nanotubes Carbon Nanostruct* 23:530–534. <https://doi.org/10.1080/1536383X.2013.870900>
- Scharff P, Ritter U, Matyshevska OP, Prylutska SV, Grynyuk II, Golub AA, Prylutsky YuI, Burlaka AP (2008) Therapeutic reactive oxygen generation. *Tumori* 94:278–283. <https://doi.org/10.1177/030089160809400221>
- Sen CK (2001) Antioxidant and redox regulation of cellular signaling: introduction. *Med Sci Sports Exerc* 33:368–370. <https://doi.org/10.1097/00005768-200103000-00005>
- Skamrova GB, Laponogov I, Buchelnikov AS, Shckorbatov YG, Prylutska SV, Ritter U, Prylutsky YI, Evstigneev MP (2014) Interceptor effect of C_{60} fullerene on the in vitro action of aromatic drug molecules. *Eur Biophys J* 43:265–276. <https://doi.org/10.1007/s00249-014-0960-2>
- Song K, Coleman RA, Xiaoyan Z, Alber C, Ballas ZK, Waldschmidt TJ, Cook RT (2002) Chronic ethanol consumption by mice results in activated splenic T cells. *J Leukoc Biol* 72:1109–1116
- Suzuki K, Ohno H, Oh-ishi S, Kizaki T, Ookawara T, Fukii J, Radak A, Taniguchi N (2000) Superoxide dismutases in exercise and disease. In: Sen CK, Packer L, Hanninen O (eds) *Handbook of oxidants and antioxidants and exercise*. Elsevier, Amsterdam, pp 243–295
- Vancampfort D, Hallgren M, Vandael H, Probst M, Van Hoof P, Firth J et al (2020) Functional exercise capacity in inpatients with alcohol use disorder versus healthy controls: a pilot study. *Alcohol* 82:47–52. <https://doi.org/10.1016/j.alcohol.2019.07.006>
- Vereshchaka IV, Bulgakova NV, Maznychenko AV, Gonchar OO, Prylutsky YuI, Ritter U, Moska W, Tomiak T, Nozdrenko DM, Mishchenko IV, Kostyukov AI (2018) C_{60} fullerenes diminish the muscle fatigue in rats comparable to *N*-acetylcysteine or β -alanine. *Front Physiol* 9:517. <https://doi.org/10.3389/fphys.2018.00517>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.



Research article

C₆₀ fullerene protective effect against the rat *muscle soleus* trauma

Dmytro Nozdrenko^a, Olexandr Motuziuk^{a,b}, Svitlana Prylutska^c,
Tetiana Matviienko^a, Kateryna Bogutska^a, Daria Franskevych^a,
Nataliya Nurishchenko^a, Olga Abramchuk^b, Yuriy Prylutskyi^{a,*}

^a ESC "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv, 01601, Ukraine

^b Faculty of Biology and Forestry, Lesya Ukrainka Volyn National University, Lutsk, 43025, Ukraine

^c Faculty of Plant Protection, Biotechnology and Ecology, National University of Life and Environmental Science of Ukraine, Kyiv, 03041, Ukraine



ARTICLE INFO

Keywords:

C₆₀ fullerene

Muscle soleus

Muscle trauma

Biomechanical parameters of muscle contraction

Blood biochemical indicators

ABSTRACT

Muscle trauma is one of the most common body injuries. Severe consequences of muscle trauma are ischemic injuries of the extremities. It is known that the intensification of free radical processes takes place in almost most acute diseases and conditions, including muscle trauma. C₆₀ fullerene (C₆₀) with powerful antioxidant properties can be considered a potential nanoagent for developing an effective therapy for skeletal muscle trauma. Here the water-soluble C₆₀ was prepared and its structural organization has been studied by the atomic force microscopy and dynamic light scattering techniques. The selective biomechanical parameters of *muscle soleus* contraction and biochemical indicators of blood in rats were evaluated after intramuscular injection of C₆₀ 1 h before the muscle trauma initiation. Analysis of the force muscle response after C₆₀ injection (1 mg kg⁻¹ dose) showed its protective effect against ischemia and mechanical injury at the level of 30 ± 2 % and 17 ± 1 %, accordingly, relative to the pathology group. Analysis of biomechanical parameters that are responsible for correcting precise positioning confirmed the effectiveness of C₆₀ at a level of more than 50 ± 3 % relative to the pathology group. Moreover, a decrease in the biochemical indicators of blood by about 33 ± 2 % and 10 ± 1 % in ischemia and mechanical injury, correspondingly, relative to the pathology group occurs. The results obtained demonstrate the ability of C₆₀ to correct the functional activity of damaged skeletal muscle.

1. Introduction

Muscle trauma is among the most common injuries to the human body [1]. The problem of determining the severity of myopathic damage continues to be of great concern due to the difficulties in making decisions regarding treatment, determining the quality of the therapy used, as well as the relatively high relapse rate. Therefore, it is necessary to understand the processes at the molecular (cellular) level inherent in a particular type of skeletal muscle damage that are involved in their healing. The most important of these is inflammation. The inflammatory response depends on two factors, namely the actual physical damage and muscle vascularization at the time of injury [2]. All this ultimately affects the immune and cytokine responses of the body and makes it difficult to adequately interpret the mechanisms of the studied pathology.

* Corresponding author.

E-mail address: prylut@ukr.net (Y. Prylutskyi).

Severe consequences of muscle injuries are ischemic injuries of the extremities. The rapid determination of the degree of ischemia that has occurred is critical for adequate treatment [1,2]. Ischemic tissue damage triggers biochemical reactions that are initiated under hypoxic conditions after a few minutes due to insufficient blood supply and continue for two to 3 h [3]. With ischemia lasting three or more hours, necrotic changes in muscle tissue (the amount of necrosis can reach up to 60 %) as well as nervous degradation occur. Activated neutrophils attracted to a lesion release free radicals, which is a characteristic manifestation of ischemic damage [3, 4].

The main clinical method for assessing the viability of muscle fibers is biochemical analysis. Such blood biochemical indicators as levels of creatine phosphokinase (CPK), creatinine, lactate dehydrogenase (LDH) and lactate (LA) are markers of muscle tissue disorders that are used in clinical studies to determine the degree of damage and evaluate therapeutic procedures [5].

The dynamics of muscle contraction is determined by specific mechanisms of interaction between motor neuron pools, entering the muscle, as well as actin and myosin myofilaments. Therefore, an adequate understanding and analysis of changes in neuromuscular pathologies require a comprehensive experimental approach with the possibility of simultaneous control of various biomechanical parameters. Only in this case does it become possible to trace changes in the response of a neuromuscular system to stimulation, which are responsible for the development of precise positional movements, the analysis of which is important in making a conclusion about the efficiency of the therapeutic agent used.

Literature data [6] indicate that free radicals are one of the main factors of muscle tissue damage. They initiate lipid peroxidation (LPO), suppress the ATPase (Adenosinetriphosphatase) activity and mitochondrial respiratory chain enzymes. Hence, antioxidant therapy, including the development of novel effective nanoscale free radical scavengers, remains an attractive strategy for skeletal muscle trauma.

C₆₀ fullerene is a fairly strong electronic acceptor capable of absorbing a large number of free radicals [7]. We have previously shown, that water-soluble C₆₀ fullerenes prevent the restraint of stress-induced oxidative disorders in rat brain and heart tissues [8] as well as an acute liver injury [9], diminish muscle fatigue in rats [10], markedly decrease the level of oxidative stress in diet-induced obesity rats [11] and, finally, realize therapeutic effects in the models of muscle injury and ischemia of the *muscle soleus* in rats [12,13]. Recently the authors [14,15] demonstrated the protective effect of C₆₀ fullerene on liver tissue in liver ischemia reperfusion injury as well as on lung and renal tissue in lower extremity ischemia-reperfusion injury in rats, which opens up a prospect for its use in biomedicine.

Taking into consideration the preliminary obtained data, this work aimed to evaluate the protective effect of C₆₀ fullerene against the rat *muscle soleus* trauma (mechanical injury and ischemia of varying severity), based on changes in the biomechanical markers of the contractile process and blood biochemical indicators.

2. Materials and methods

2.1. Preparation and characterization of C₆₀FAS

C₆₀ fullerene aqueous solution (C₆₀FAS) was prepared by transferring C₆₀ molecules from toluene into an aqueous dispersion using an ultrasonic bath [16]. The obtained C₆₀FAS (0.15 mg ml⁻¹) was stable for 18 months at a temperature +4 °C.

The structural organization of C₆₀FAS was investigated by the atomic force microscopy (AFM) and dynamic light scattering (DLS) methods [17]. AFM and DLS measurements were performed on the systems "Solver Pro M" (NT-MDT) and Zetasizer Nano ZS (Malvern Ins. Ltd), correspondingly, at room temperature.

2.2. Animals

Male Wistar rats (170 ± 5 g, 3-month-old) were used in the study. The experimental animals were housed in Plexiglas cages and kept in an air-filtered and temperature-controlled (21 ± 1 °C) room under 12-h light/12-h dark conditions. Rats received a standard pellet diet and water *ad libitum*. All procedures complied with the ARRIVE guidelines. The use of the laboratory animals was approved by the Biomedical Ethics Committee of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv (protocol No. 2 dated September 2, 2022) and performed in accordance with the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and Article 26 of the Law of Ukraine "On the Protection of Animals from Cruelty" (No. 3447-IV, 21.02.2006), as well as European Union Directive of 22 September 2010 (2010/63/EU) for the protection of animals used for scientific purposes.

The animals were randomly divided into the following experimental groups:

- control group (n = 7);
- pathology (non-treated) groups (muscle injury and ischemia with varying degrees of severity (1, 2, and 3), respectively, without drug administration) (n = 7 in each group);
- pathology (C₆₀-treated) groups (intramuscular administration of C₆₀FAS (1 and 2 mg kg⁻¹ doses) 1 h before muscle injury and ischemia with different degrees of severity (1, 2 and 3)) (n = 7 in each group).

2.3. Biomechanical and biochemical analyzes

Muscle soleus was released from the surrounding tissues. Its tendon was cut transversely in the distal part, and ventral roots - directly

at their exit points from the spinal cord. Efferents were stimulated by 2 ms (millisecond) electrical pulses generated by a pulse generator. Force of muscle contraction was measured by using strain gauges [12,13].

For muscle ischemization [12], the branch of the femoral artery that provides blood supply to the experimental muscle was tied with ligatures. The duration of ischemization was 1, 2, and 3 h (1, 2, and 3 degrees of ischemia, respectively).

Muscle injury was induced by compressing the muscle for 1, 2, and 3 min (1, 2, and 3 degrees of injury severity, respectively) with a 3.5 mg cm^{-2} pressure clamp [18].

Analyzing the myotic response of the muscle under study while applying a modulated stimulating signal, we analyzed several basic biomechanical parameters as markers of the muscle dysfunctions [12,13,19], namely:

1. Change in the level of generation of minimum contraction force ($F_{\min}$). This marker is an indicator of the maximum changes in a muscle caused by a pathological process;
2. Change in the time to reach the maximum force response ($t_{\max}$). This marker indicates the level of muscle dysfunction when it performs maximum strength tasks;
3. Change in the level of generation of maximum contraction force ($F_{\max}$). This marker is an indicator of general muscle dysfunction during the development of pathology;
4. Change in the integrated power of muscle contraction (S). This marker, as a calculated area under the force curve, is an indicator of muscle overall performance;
5. Change in the time of reduction of muscle contraction force by 50 % from the initial level (t_{50}). This indicator is used to assess the development of muscle fatigue;
6. Change in the time of force parameters output to the initial level (t_0). This indicator describes a change in the rigid structure of the muscle during the development of pathology;
7. Change in muscle force response onset time (change in latent period; t_{start}). Using this indicator one can evaluate the level of pathology at the stages of the interaction of muscle myofilaments, calcium pump dysfunction, and sarcoplasmic reticulum system.

The levels of enzyme content (CPK and LDH) as well as creatinine, LA, reduced glutathione (GSH), hydrogen peroxide (H_2O_2), thiobarbituric acid reactive substances (TBARS), and catalase (CAT) activity in the blood plasma of experimental animals, as markers of muscle trauma [20], were determined using clinical biochemical equipment (Erba, Czech Republic).

2.4. Statistics

The statistical analysis of the obtained results was performed by using the Statistica 13.3 software. Each of the experimental force curves is the result of averaging 10 similar tests. Each biochemical measurement was carried out at least three times. A factorial ANOVA analysis with Tukey *post-hoc* test was used depending on such factors of variation as dose and degree of muscle trauma severity. Data are presented as the means $\pm$ SEM for each group. Differences between groups were considered significant at the level of $p < 0.05$.

3. Results and discussion

3.1. Structural organization of C_{60}FAS

Because specific biological effects are highly dependent on particle size [21,22], we have investigated the size of C_{60} fullerene nanoparticles in a created aqueous solution using the AFM and DLS techniques.

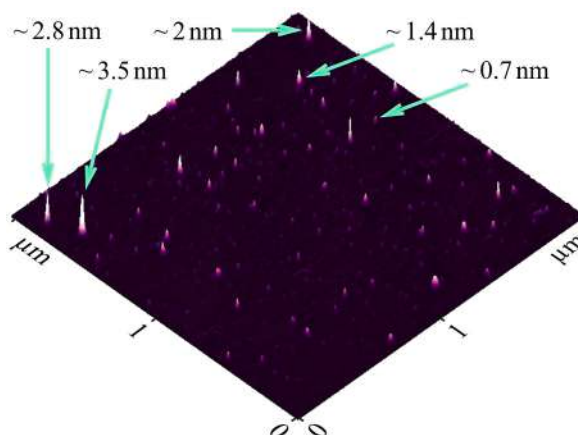


Fig. 1. AFM image of C_{60} fullerene nanoparticles present in C_{60}FAS (0.15 mg ml^{-1}). Values near arrows indicate the height of the nanoobjects.

Fig. 1 demonstrates the 3D AFM image of C_{60} fullerene nanoparticles present in C_{60} FAS, namely a single C_{60} molecule (~ 0.7 nm (nanometer)) and its nanoclusters (~ 1.4 , ~ 2.0 , ~ 2.8 , and ~ 3.5 nm).

Fig. 2 shows the DLS data for studied C_{60} FAS. As one can see C_{60} FAS also contains big C_{60} fullerene nanoclusters with following hydrodynamic size ranging: ~ 64 (8 %), ~ 75 (32 %), ~ 82 (42 %) and ~ 106 (18 %) nm.

3.2. Biomechanics of muscle soleus contraction

The crush syndrome applied as a factor of muscle injury [18] caused a significant decrease in the *muscle soleus* force response (Fig. 3).

Thus, there was a sharp decrease in the *muscle soleus* force response as early in the first seconds of stimulation with a subsequent decrease to 43 ± 3 %, 38 ± 2 %, and 19 ± 2 % in cases of degree 1, 2 and 3 muscle injury, respectively, relative to the control group. The administration of C_{60} FAS (1 mg kg^{-1} dose) increased the muscle force response by 21 ± 2 %, 19 ± 1 %, and 12 ± 1 % for degrees 1, 2, and 3 of muscle injury, respectively, relative to the pathology group. Increasing the C_{60} FAS dose (2 mg kg^{-1}) further marginally increased the strength response by 10 ± 1 %.

After initiation of ischemic injury, the contractile force of the rat *muscle soleus* decreased to 71 ± 4 %, 63 ± 3 %, and 38 ± 3 % in cases of the 1st, 2nd and 3rd degree of ischemia, respectively, relative to the control group (Fig. 3). C_{60} FAS injections increased the *muscle soleus* force response by 29 ± 2 %, 23 ± 2 %, and 37 ± 2 % at the dose of 1 mg kg^{-1} in cases of the 1, 2 and 3 degree of ischemia, respectively, relative to the pathology group and additionally by 10 ± 1 % at the dose of 2 mg kg^{-1} .

Thus, the applied dose of C_{60} FAS injection of 1 mg kg^{-1} can correct the force response of the muscle with a mechanical injury and ischemia of varying severity. Doubling the dose of the protective agent did not significantly change muscle performance.

The use of biomechanical markers of amplitude-velocity changes in the force response to analyzing the pathological processes under study has revealed significant qualitative differences between them in the C_{60} FAS applied (Figs. 4 and 5). It turned out that these biomechanical markers of muscle contraction had different sensitivity to the severity of the developing pathology and lined up in the following order: $F_{\min} \rightarrow t_{\max} \rightarrow F_{\max} \rightarrow S \rightarrow t_{50} \rightarrow t_0 \rightarrow t_{\text{start}}$.

For example, the change in minimum contraction force ($F_{\min}$) with a muscle injury was 31 ± 2 % and 17 ± 1 % in cases of the 1 and 2 degrees of pathology, respectively, relative to the control group (Fig. 4). The protective effect of C_{60} FAS (dose 1 mg kg^{-1}) on this marker was 60 ± 3 %, 23 ± 1 %, and 18 ± 1 % in cases of the 1, 2, and 3 degrees of pathology, respectively, relative to the pathology group. The change in the time to reach maximum muscle contraction force ($t_{\max}$) with a muscle injury was 42 ± 2 % and 21 ± 1 % in cases of the 1 and 2 degrees of pathology, respectively, relative to the control group (Fig. 3). The protective effect of C_{60} FAS (1 mg kg^{-1} dose) on this marker was 67 ± 3 %, 38 ± 1 %, and 21 ± 1 % in cases of the 1, 2, and 3 degrees of pathology, respectively, relative to the pathology group. The $F_{\max}$, S , and t_{50} markers showed the same tendency to change with C_{60} FAS (1 mg kg^{-1} dose) application in cases of the 1, 2, and 3 degrees of muscle injury (Fig. 3). At the same time, markers t_0 and t_{start} changed only in case of 2 and 3 degrees of the pathological process (76 ± 3 % and 49 ± 2 % of the control group) and recovered by $(92-95) \pm 3$ % when C_{60} FAS (1 mg kg^{-1} dose) was applied (Fig. 3).

When the C_{60} FAS dose (2 mg kg^{-1}) was increased, all of the markers tested responded positively: from 35 ± 2 % for the most sensitive marker $F_{\min}$ to 10 ± 1 % for the least sensitive marker t_{start} relative to the pathology group (Fig. 4).

Qualitative changes in muscle dynamics can be traced more significantly when analyzing the protective effect of C_{60} FAS injection on biomechanical markers of muscle contraction during the development of ischemia of varying severity (Fig. 5). So, for example, the change in the minimum contraction force ($F_{\min}$) was 60 ± 3 % and 47 ± 2 % in the case of 1 and 2 degrees of pathology development, respectively, relative to the control. The effect of C_{60} FAS at a 1 mg kg^{-1} dose on this marker was 65 ± 3 %, 56 ± 3 %, and 28 ± 1 % in cases of the 1, 2, and 3 degrees of pathology, respectively, relative to the pathology group. It should also be noted that C_{60} FAS effect is enhanced by $(25-30) \pm 2$ % for the first three sensitive markers ($F_{\min}$, $t_{\max}$, and $F_{\max}$) when the dose is increased to 2 mg kg^{-1} .

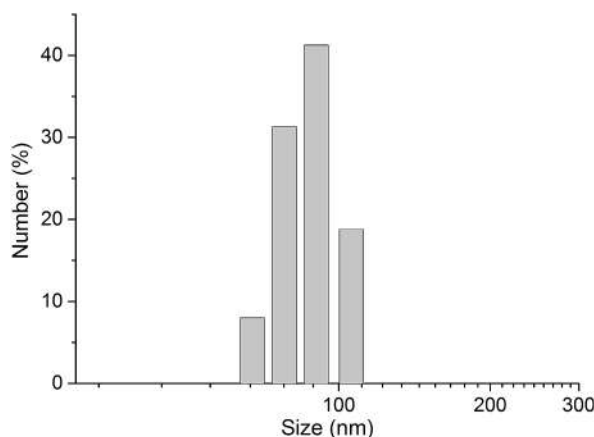


Fig. 2. Distribution of the number of light scattering particles present in C_{60} FAS (0.15 mg ml^{-1}) (in %) according to their hydrodynamic size.

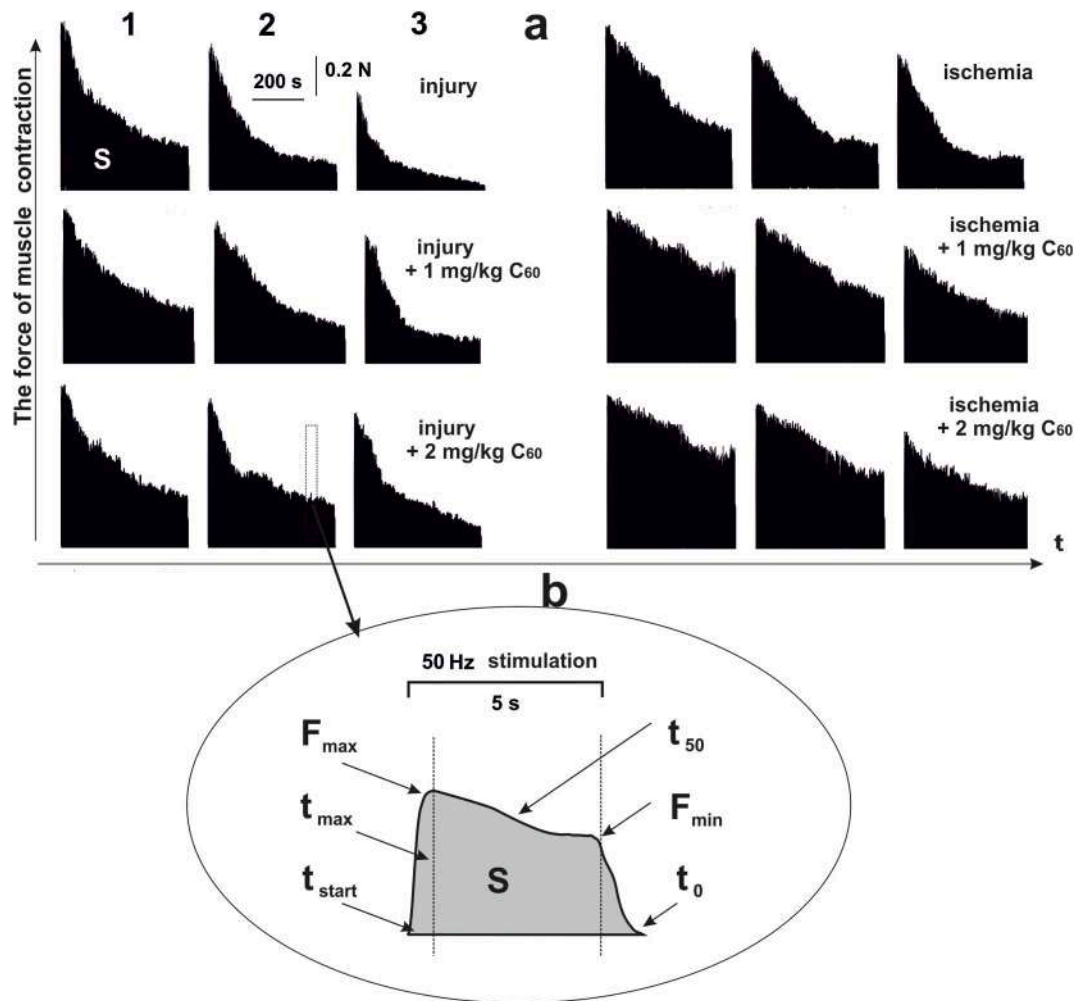


Fig. 3. Force of *muscle soleus* contraction during stimulation at a frequency of 50 Hz for 5 s, duration 500 s without relaxation (a): injury - mechanical injury; ischemia - ischemic muscle; injury/ischemia +1 mg kg⁻¹ C₆₀ and injury/ischemia +2 mg kg⁻¹ C₆₀ - corresponding pathology against the background of C₆₀FAS injections at doses 1 and 2 mg kg⁻¹, respectively; 1, 2, 3 - the degree of the pathological process in the muscle. Biomechanical parameters of muscle contraction (b): S - integrated muscle power; $F_{\max}$ and $F_{\min}$ - maximum and minimum forces of a single contraction; $t_{\max}$ - time to reach maximum muscle contraction force; t_{50} - time of reduction of muscle contraction force by 50 % from the initial level; t_0 - time of force parameters output to the initial level; t_{start} - time between stimulation start and contractile process start.

Summarizing, one can assume that analysis of selected biomechanical markers in such chronological order of their maximum change makes it possible to determine the severity degree of muscle pathology and the efficiency of the therapy used, which is of paramount importance in emergency conditions.

3.3. Blood plasma biochemical indicators of rats

The analysis of blood biochemical markers, in particular, the levels of creatinine, CPK, LA, and LDH in rat blood plasma (Fig. 6), makes it possible to evaluate physiological changes in the muscle and the therapeutic effect of the drug on pathological processes. As can be seen, they all increase with increasing time after the initiation of injury, which indicates that the muscular system is performing super-intense work for its physiological level, followed by the development of muscle fatigue.

Change in CPK concentration is one of the important markers of pathological processes in skeletal muscles. With mechanical damage to the muscles, there is a release of the CPK from the cells and increase its concentration in the blood. The increase in the blood CPK fraction (Fig. 6a) from 500 ± 15 Units l⁻¹ in the control group to 1490 ± 21 , 1622 ± 26 , and 2430 ± 29 Units l⁻¹ in cases of grade 1, 2, and 3 muscle injury, respectively, is the result of myocyte wall destruction with partial release of intramyocyte enzymes into the extracellular space [23]. When C₆₀FAS (1 mg kg⁻¹ dose) was injected, CPK levels decreased by 10 ± 1 % in cases of the 1, 2, and 3 degrees of muscle injury, respectively, relative to the pathology group and did not significantly change when the protective dose of C₆₀FAS was increased to 2 mg kg⁻¹.

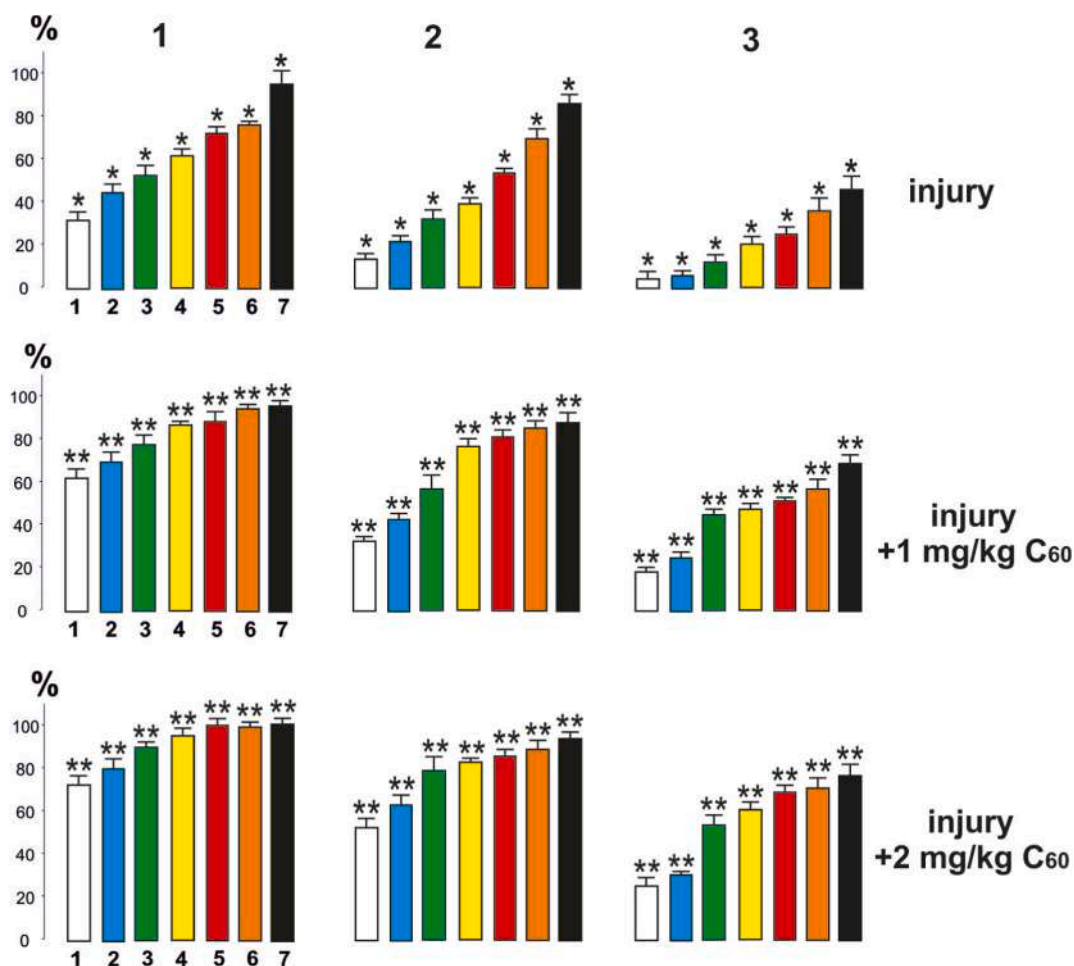


Fig. 4. Biomechanical parameters of muscle contraction (1 - $F_{\min}$; 2 - $t_{\max}$; 3 - $F_{\max}$; 4 - S ; 5 - t_{50} ; 6 - t_0 ; 7 - t_{start}) in the order of their maximum change in the analysis of mechanical injury (in % relative to control): injury + $1 \text{ mg kg}^{-1} \text{ C}_{60}$ and injury + $2 \text{ mg kg}^{-1} \text{ C}_{60}$ - mechanical injury against the background of C_{60}FAS injections at doses of 1 and 2 mg kg^{-1} , accordingly; 1, 2, 3 - degree of muscle injury. * $p < 0.05$ vs. control group; ** $p < 0.05$ vs. muscle injury group.

During the development of the inflammatory reactions after the initiation of injury, a significant depletion of cellular energy substances (especially ATP) take places, which causes loss of the ion gradient through cell membranes. This leads to the acidification of the pH of intra- and extracellular media [19]. Fig. 6b shows that this parameter increased from $10.3 \pm 0.5 \text{ M}$ in the control group to 15.7 ± 1 , 17.9 ± 1 , and $22.4 \pm 1 \text{ M}$ in cases of the 1, 2, and 3 degrees of muscle injury, respectively. When injected with C_{60}FAS (1 mg kg^{-1} dose), the LA level decreased by $11 \pm 1 \%$ in the cases of 1, 2, and 3 degrees of muscle injury, respectively, relative to the pathology group and did not significantly change with an increase in the protective dose of C_{60}FAS up to 2 mg kg^{-1} .

The level of LDH enzyme allows us to assess the general performance status of the damaged muscle after its prolonged activation. The change in the level of LDH from $225 \pm 12 \text{ Units l}^{-1}$ in the control group to 510 ± 14 , 593 ± 9 , and $886 \pm 15 \text{ Units l}^{-1}$ after the initiation of the 1, 2, and 3 degrees of muscle injury indicates the significant muscle dysfunctions associated with excess fatigue pathogens. With C_{60}FAS injection (1 mg kg^{-1} dose), LDH levels decreased by $10 \pm 1 \%$ in the cases of 1, 2, and 3 degrees of muscle injury, respectively, relative to the pathology group and did not significantly change when the protective dose of C_{60}FAS was increased to 2 mg kg^{-1} (Fig. 6b).

The relatively low protective effect of C_{60}FAS ($(10\text{--}14) \pm 1 \%$), revealed by the analysis of changes in blood biochemical indicators, is due to the fact that injury with a muscle tissues rupture is the most severe pathology, covering not only muscle structures but also complicated by high-level pain symptoms. Therefore, the usage of C_{60}FAS , in our opinion, is not a sufficiently complete solution for an adequate therapeutic model. So, the previously discovered synergism of the action of menthol as an analgesic agent and C_{60}FAS on the post-traumatic process of restoring skeletal muscle functions opens up prospects for the clinical application of such combination therapy [13].

Analysis of blood biochemical markers in case of ischemic injuries revealed a significant C_{60} fullerene protective effect. So, when C_{60}FAS (1 mg kg^{-1} dose) was injected, the creatinine level decreased by $31 \pm 2 \%$, $28 \pm 2 \%$, and $21 \pm 1 \%$ in the case of 1, 2, and 3 degrees of ischemia, respectively, relative to the pathology group. Increasing the C_{60}FAS dose (2 mg kg^{-1}) additionally improved this

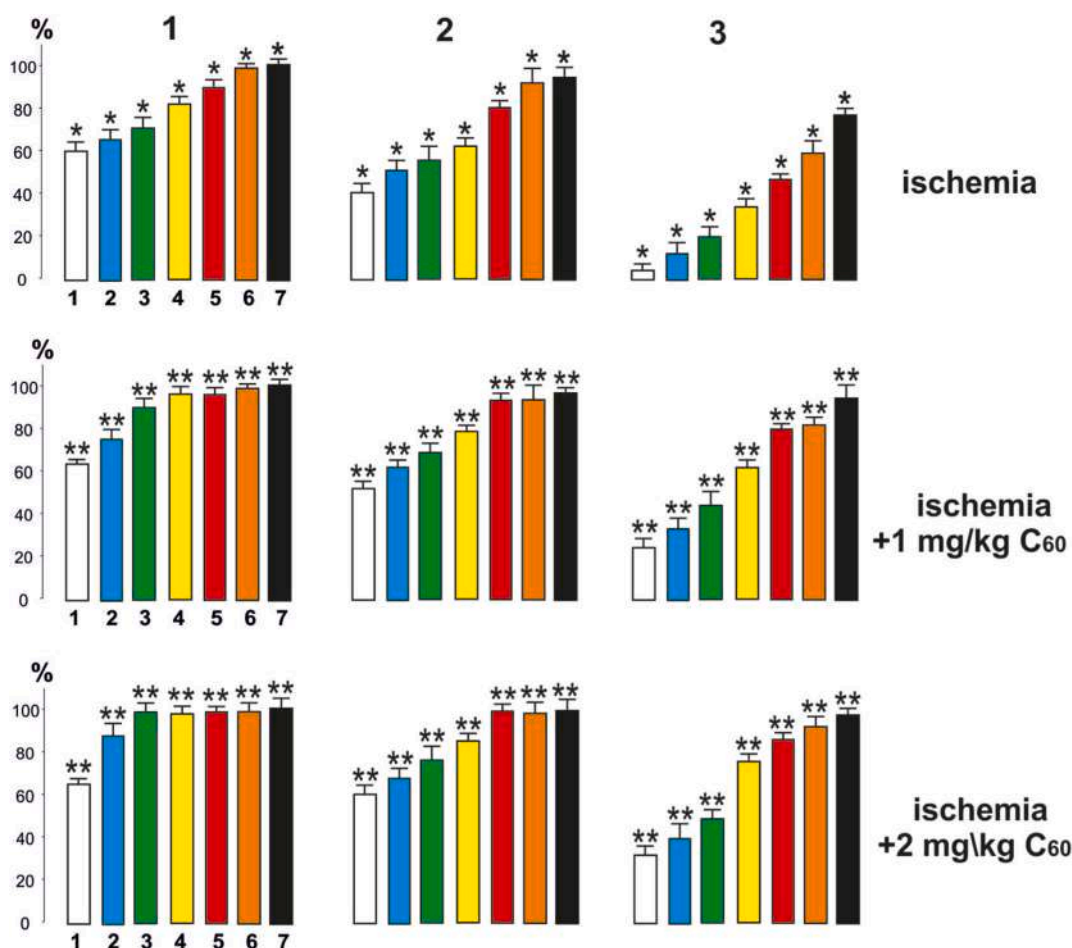


Fig. 5. Biomechanical parameters of muscle contraction (1 - F_{min} ; 2 - t_{max} ; 3 - F_{max} ; 4 - S ; 5 - t_{50} ; 6 - t_0 ; 7 - t_{start}) in the order of their maximum change in ischemia analysis (in % relative to control): ischemia +1 mg kg⁻¹ C₆₀ and ischemia +2 mg kg⁻¹ C₆₀ - muscle ischemia against the background of C₆₀FAS injection at 1 and 2 mg kg⁻¹ doses, accordingly; 1, 2, 3 - degree of muscle ischemia. * p < 0.05 vs. control group; ** p < 0.05 vs. ischemia group.

parameter by an average of 10 ± 1 % (Fig. 6a).

With C₆₀FAS injection (1 mg kg⁻¹ dose), the CPK level decreased by 29 ± 1 %, 48 ± 3 %, and 23 ± 1 % in the case of 1, 2, and 3 degrees of ischemia, respectively, relative to the pathology group. Increasing the C₆₀FAS dose (2 mg kg⁻¹) additionally improved this parameter by an average of 10 ± 1 % (Fig. 6a).

When C₆₀FAS (1 mg kg⁻¹ dose) was injected, the LA level decreased by 46 ± 3 %, 49 ± 3 %, and 35 ± 2 % in the case of 1, 2, and 3 degrees of ischemia, respectively, relative to the pathology group. Increasing the C₆₀FAS dose (2 mg kg⁻¹) additionally improved this parameter by an average of 13 ± 1 % (Fig. 6b).

With C₆₀FAS injection (1 mg kg⁻¹ dose), the LDH level decreased by 47 ± 3 %, 34 ± 2 %, and 26 ± 2 % in the cases of 1, 2, and 3 degrees of ischemia, respectively, relative to the pathology group. Increasing the C₆₀FAS dose (2 mg kg⁻¹) additionally improved this parameter by an average of 10 ± 1 % (Fig. 6b).

Thus, a clear tendency to decreasing the described biochemical blood parameters in the case of ischemic muscle injuries by about $(37-56) \pm 2$ % relative to the pathology group when using C₆₀FAS in a protective regimen takes place. This may indicate activation of the endogenous antioxidant system by C₆₀ fullerene nanoparticles, which is confirmed by previously obtained data [9-13,19].

The inflammatory process that occurs immediately after a muscle injury is a source of free radicals and contributes to the LPO intensification [13]. As a result of biochemical tests, we evaluated the indicators of pro- and antioxidant balance (CAT, H₂O₂, TBARS, and GSH) in the blood plasma of rats after the muscle trauma initiation (Fig. 7).

C₆₀FAS injection (1 mg kg⁻¹ dose) reduced CAT activity by 10 ± 1 % for muscle injury, and by 33 ± 1 %, 47 ± 2 %, and 22 ± 1 % for ischemia with a grade 1, 2, and 3 pathologies, respectively, relative to the pathology group. Increasing the C₆₀FAS dose (2 mg kg⁻¹) did not significantly change this index for muscle injury and further improved it by 10 ± 1 % for ischemia (Fig. 7a).

The concentrations of H₂O₂ after C₆₀FAS injection (1 mg kg⁻¹ dose) decreased by 10 ± 1 % for muscle injury and ischemia - by 29 ± 1 %, 35 ± 1 %, and 22 ± 1 % in the case of 1, 2, and 3 degrees of pathology development, respectively, relative to the pathology

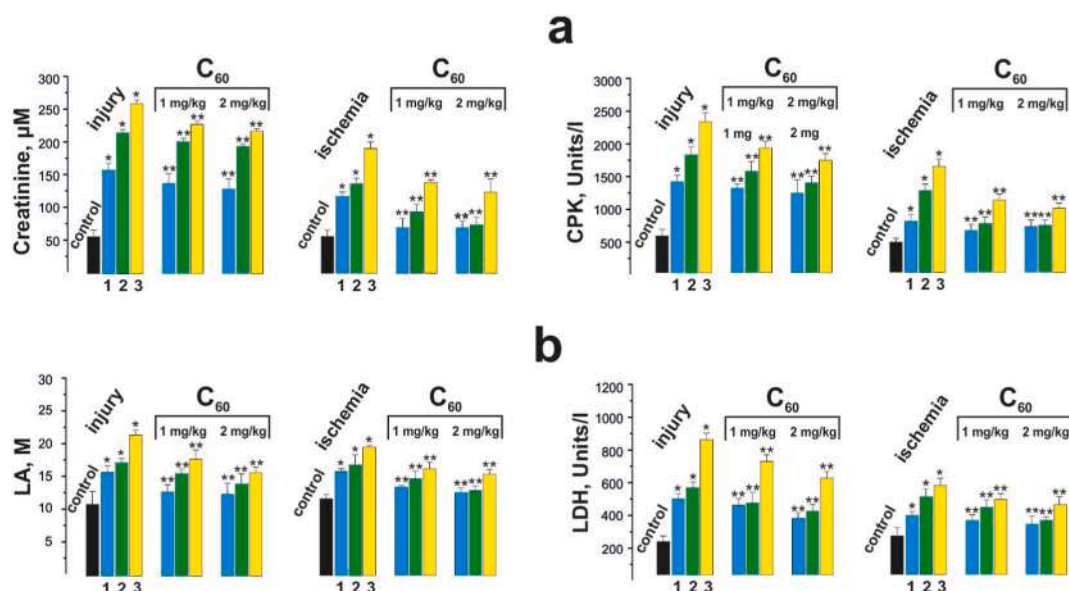


Fig. 6. Biochemical indicators (creatinine and CPK (a), LA and LDH (b) in blood plasma) of the development of pathological processes in the *muscle soleus* of rats: injury - mechanical injury; ischemia - ischemic muscle; C_{60} - the corresponding pathology on the background of C_{60}FAS injection at 1 and 2 mg kg^{-1} doses, accordingly; 1, 2, 3 - the degree of the pathological process development in the muscle; * $p < 0.05$ vs. control group; ** $p < 0.05$ vs. the corresponding pathology group.

group. Increasing the C_{60}FAS dose (2 mg kg^{-1}) did not significantly change this parameter for muscle injury and additionally improved it by $11 \pm 1 \%$ for ischemia (Fig. 7a).

The concentrations of TBARS after C_{60}FAS injection (1 mg kg^{-1} dose) decreased by $10 \pm 1 \%$ for muscle injury and ischemia - by $33 \pm 2 \%$, $38 \pm 2 \%$, and $27 \pm 1 \%$ in the case of the 1, 2, and 3 degrees of pathology development, respectively, relative to the pathology group. Increasing the C_{60}FAS dose (2 mg kg^{-1}) did not significantly change this parameter for muscle injury and additionally improved it by $10 \pm 1 \%$ for ischemia (Fig. 7b).

GSH concentrations were reduced by $11 \pm 1 \%$ for muscle injury and by $29 \pm 1 \%$, $33 \pm 2 \%$, and $25 \pm 1 \%$ for ischemia in the case of the 1, 2 and 3 pathology development, respectively, relative to the pathology group when C_{60}FAS (1 mg kg^{-1} dose) was injected. Increasing the C_{60}FAS dose (2 mg kg^{-1}) did not significantly change this parameter for muscle injury and further improved it by $10 \pm 1 \%$ for ischemia (Fig. 7b).

Thus, the protective administration of C_{60}FAS reduces the described biochemical parameters for ischemic injuries by $(36\text{--}43) \pm 2 \%$, and for muscular injuries by $(10\text{--}13) \pm 1 \%$ relative to the pathology group.

Summarizing, the positive effect of C_{60} fullerene injections on the studied muscle pathologies is associated with its powerful antioxidant properties [24]. By inactivation of the free radicals during the development of skeletal muscle trauma, the proposed protective therapy using C_{60} fullerene reduces the degree of its damage.

4. Conclusion

The obtained results indicate that the C_{60}FAS has a pronounced protective effect on the resumption of muscle functional activity after the occurrence of pathological processes associated with muscle trauma. In particular, the analysis of the force muscle response after C_{60}FAS injection (1 mg kg^{-1} dose) showed its protective effect against ischemia and mechanical injury at the level of $30 \pm 2 \%$ and $17 \pm 1 \%$, accordingly, relative to the pathology group. Increasing the C_{60}FAS dose (2 mg kg^{-1}) did not significantly increase in the protective effect for both injuries. The analysis of the biomechanical markers responsible for the correction of precise positioning during protective exposure to C_{60}FAS confirmed its effectiveness at the level of more than $50 \pm 3 \%$ relative to the pathology group. The use of C_{60}FAS (1 mg kg^{-1} dose) showed a clear tendency to decrease the studied biochemical blood parameters by about $33 \pm 2 \%$ and $10 \pm 1 \%$ in ischemia and muscle injury, correspondingly, relative to the pathology group. Increasing the C_{60}FAS dose (2 mg kg^{-1}) leads to a reliable increase in the protective effect by another $10 \pm 1 \%$ for ischemia and did not significantly change for muscle injury.

Thus, C_{60} fullerenes influence the activity of endogenous antioxidants, preventing the occurrence of dysfunction in the active muscle and, therefore, maintaining it within the physiological norm throughout the contraction process. This opens up the possibility of their usage as potential protective nanoagents capable of correcting the functional muscle activity during its mechanical injury and most efficiently affecting the resumption of the precise control of motor reactions system.

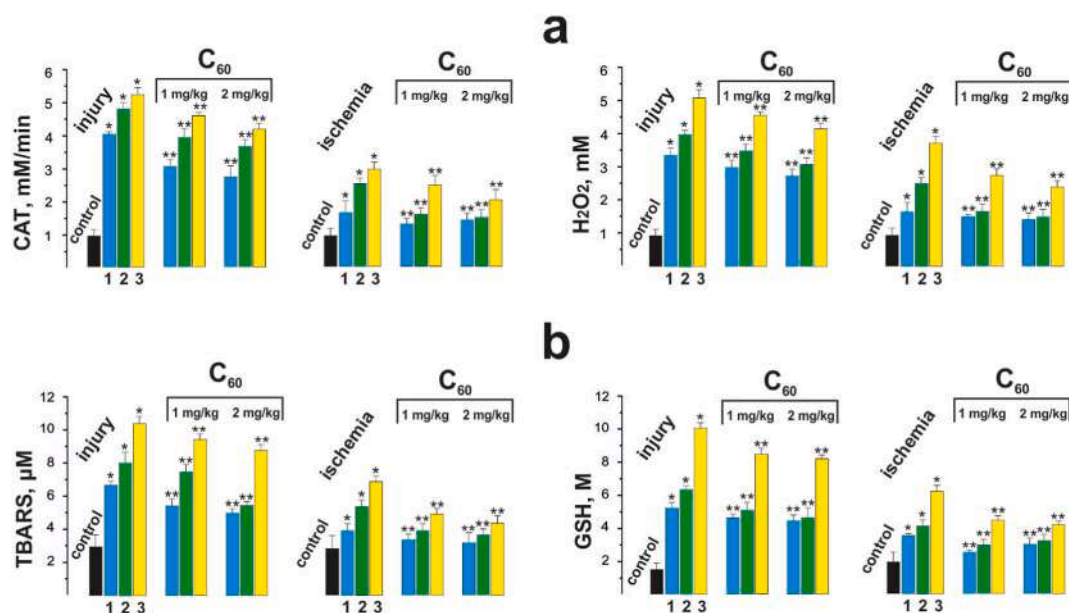


Fig. 7. The prooxidant-antioxidant balance indicators (CAT and H₂O₂ (a), TBARS and GSH (b)) in the blood plasma of rats: injury - mechanical injury; ischemia - ischemic muscle; C₆₀ - the corresponding pathology on the background of injections of C₆₀FAS at doses of 1 and 2 mg kg⁻¹, accordingly; 1, 2, 3 - the degree of development of the pathological process in the muscle; **p* < 0.05 vs. control group; ***p* < 0.05 vs. the corresponding pathology group.

Funding statement

This work was supported by the NRFU (2022.01/0004).

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Dmytro Nozdrenko: Writing – original draft, Methodology, Investigation, Funding acquisition. **Olexandr Motuziuk:** Investigation. **Svitlana Prylutska:** Methodology, Investigation. **Tetiana Matvienko:** Investigation. **Kateryna Bogutskaya:** Investigation. **Daria Franskevych:** Investigation. **Nataliya Nurishchenko:** Investigation. **Olga Abramchuk:** Investigation. **Yuriy Prylutsky:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgments

The authors are grateful to Prof. U. Ritter (Technical University of Ilmenau, Germany) for kindly providing samples (C₆₀FAS) for research.

References

- Hamilton, J.-M. Alonso, T.M. Best, Time for a paradigm shift in the classification of muscle injuries, *J. Sport Health Sci.* 6 (2017) 255–261, <https://doi.org/10.1016/j.jshs.2017.04.011>.
- Smith, M.J. Kruger, R.M. Smith, K.H. Myburgh, The inflammatory response to skeletal muscle injury: illuminating complexities, *Sports Med.* 38 (2008) 947–969, <https://doi.org/10.2165/00007256-200838110-00005>.

- [3] M. Murdock, M.M. Murdoch, Compartment syndrome: a review of the literature, *Clin. Podiatr. Med. Surg.* 29 (2012) 301–310, <https://doi.org/10.1016/j.cpm.2012.02.001>.
- [4] S.K. Bortolotto, W.A. Morrison, A. Messina, The role of mast cells and fiber type in ischemia reperfusion injury of murine skeletal muscles, *J. Inflamm.* 1 (2004) 2, <https://doi.org/10.1186/1476-9255-1-2>.
- [5] S.B.P. Charge, M.A. Rudnicki, Cellular and molecular regulation of muscle regeneration, *Physiol. Rev.* 84 (2004) 209–238, <https://doi.org/10.1152/physrev.00019.2003>.
- [6] H. Amani, R. Habibey, S.J. Hajmiresmail, S. Latifi, H. Pazoki-Toroudi, O. Akhavan, Antioxidant nanomaterials in advanced diagnoses and treatments of ischemia reperfusion injuries, *J. Mater. Chem. B* 5 (2017) 9452–9476, <https://doi.org/10.1039/c7tb01689a>.
- [7] S. Goodarzi, T. Da Ros, J. Conde, F. Sefat, M. Mozafari, Fullerene: biomedical engineers get to revisit an old friend, *Mater. Today* 20 (2017) 460–480, <https://doi.org/10.1016/j.mattod.2017.03.017>.
- [8] O.O. Gonchar, A.V. Maznychenko, N.V. Bulgakova, I.V. Vereshchaka, T. Tomiak, U. Ritter, Y.I. Prylutsky, I.M. Mankovska, A.I. Kostyukov, C₆₀ fullerene prevents restraint stress-induced oxidative disorders in rat tissues: possible involvement of the Nrf2/ARE-antioxidant pathway, *Oxid. Med. Cell. Longev.* 2018 (2018) 2518676, <https://doi.org/10.1155/2018/2518676>.
- [9] T.I. Halenova, I.M. Varenjuk, N.M. Roslova, M.E. Dzerzhynsky, O.M. Savchuk, L.I. Ostapchenko, Yul. Prylutsky, U. Ritter, P. Scharff, Hepatoprotective effect of orally applied water-soluble pristine C₆₀ fullerene against CCl₄-induced acute liver injury in rats, *RSC Adv.* 102 (2016) 100046–100055.
- [10] I.V. Vereshchaka, N.V. Bulgakova, A.V. Maznychenko, O.O. Gonchar, Y.I. Prylutsky, U. Ritter, W. Moska, T. Tomiak, D.M. Nozdrenko, I.V. Mishchenko, A. I. Kostyukov, C₆₀ fullerenes diminish muscle fatigue in rats comparable to N-acetylcysteine or β -Alanine, *Front. Physiol.* 9 (2018) 517, <https://doi.org/10.3389/fphys.2018.00517>.
- [11] T. Halenova, N. Raksha, T. Vovk, O. Savchuk, L. Ostapchenko, Yu Prylutsky, O. Kyzyma, U. Ritter, P. Scharff, Effect of C₆₀ fullerene nanoparticles on the diet-induced obesity in rats, *Int. J. Obes.* 42 (2018) 1987–1998, <https://doi.org/10.1038/s41366-018-0016-2>.
- [12] D.M. Nozdrenko, D.O. Zavadovsky, T.Yu Matvienko, S.Yu Zay, K.I. Bogutska, Yul. Prylutsky, U. Ritter, P. Scharff, C₆₀ fullerene as promising therapeutic agent for the prevention and correction of functioning skeletal muscle at ischemic injury, *Nanoscale Res. Lett.* 12 (2017) 115, <https://doi.org/10.1186/s11671-017-1876-4>.
- [13] D. Nozdrenko, T. Matvienko, O. Vygovska, V. Soroca, K. Bogutska, A. Zholos, P. Scharff, U. Ritter, Yu Prylutsky, Post-traumatic recovery of muscle soleus in rats is improved via synergistic effect of C₆₀ fullerene and TRPM8 agonist menthol, *Appl. Nanosci.* 12 (2022) 467–478, <https://doi.org/10.1007/s13204-021-01703-z>.
- [14] A. Yavuz, A.T. Tuna, C. Ozdemir, T. Mortas, A. Küçük, E. Kasapbaşı, M. Arslan, M. Kavutçu, Ö. Kurtipek, Effects of fullerene C₆₀ on liver tissue in liver ischemia reperfusion injury in rats undergoing sevoflurane anesthesia, *Libyan J. Med.* 18 (1) (2023) 2281116, <https://doi.org/10.1080/19932820.2023.2281116>.
- [15] A.H. Arpacı, Z. Koksall, Z. Yigman, A. Kucuk, V. Sivgin, M. Arslan, M. Kavutcu, S.O.A. Dizakar, Effect of fullerenol C₆₀ on lung and renal tissue in lower extremity ischemia-reperfusion injury in sevoflurane-treated rats, *Mol. Med. Rep.* 29 (3) (2024) 54, <https://doi.org/10.3892/mmr.2024.13178>.
- [16] Yul. Prylutsky, V.M. Yashchuk, K.M. Kushnir, A.A. Golub, V.A. Kudrenko, S.V. Prylutska, I.I. Grynyuk, E.V. Buzaneva, P. Scharff, T. Braun, O.P. Matyshevska, Biophysical studies of fullerene-based composite for bio-nanotechnology, *Mater. Sci. Eng. C* 23 (2003) 109–111, [https://doi.org/10.1016/S0928-4931\(02\)00244-8](https://doi.org/10.1016/S0928-4931(02)00244-8).
- [17] S.V. Prylutska, S.V. Politenkova, K.S. Afanasieva, V.F. Korolovych, K.I. Bogutska, A.V. Sivolob, L.M. Skivka, M.P. Evstigneev, V.V. Kostjukov, Yul. Prylutsky, U. Ritter, A nanocomplex of C₆₀ fullerene with cisplatin: design, characterization and toxicity, *Beilstein J. Nanotechnol.* 8 (2017) 1494–1501, <https://doi.org/10.3762/bjnano.8.149>.
- [18] J. de Souza, C. Gottfried, Muscle injury: review of experimental models, *J. Electromyogr. Kinesiol.* 23 (2013) 1253–1260, <https://doi.org/10.1016/j.jelekin.2013.07.009>.
- [19] D. Nozdrenko, O. Abramchuk, S. Prylutska, O. Vygovska, V. Soroca, K. Bogutska, S. Khrapatyi, Yu Prylutsky, P. Scharff, U. Ritter, Analysis of biomechanical parameters of muscle soleus contraction and blood biochemical parameters in rat with chronic glyphosate intoxication and therapeutic use of C₆₀ fullerene, *Int. J. Mol. Sci.* 22 (2021) 4977, <https://doi.org/10.3390/ijms22094977>.
- [20] P. Brancaccio, G. Lippi, N. Maffulli, Biochemical markers of muscular damage, *Clin. Chem. Lab. Med.* 48 (2010) 757–767, <https://doi.org/10.1515/CCLM.2010.179>.
- [21] S. Prabha, G. Arya, R. Chandra, B. Ahmed, S. Nimesh, Effect of size on biological properties of nanoparticles employed in gene delivery, *Art. Cells Nanomed. Biotechnol.* 44 (2016) 83–91, <https://doi.org/10.3109/21691401.2014.913054>.
- [22] M.I. Melnyk, I.V. Ivanova, D.O. Dryn, Yul. Prylutsky, V.V. Hurmach, M. Platonov, L.T. Al Kury, U. Ritter, A.I. Soloviev, A.V. Zholos, C₆₀ fullerenes selectively inhibit BK_{Ca} but not K_v channels in pulmonary artery smooth muscle cells, *Nanomed. Nanotechnol. Biol. Med.* 19 (2019) 1–11, <https://doi.org/10.1016/j.nano.2019.03.018>.
- [23] H. Hagberg, Intracellular pH during ischemia in skeletal muscle: relationship to membrane potential, extracellular pH, tissue lactic acid and ATP, *Pflügers Archiv* 404 (1985) 342–347.
- [24] S.V. Eswaran, Water soluble nanocarbon materials: a panacea for all? *Curr. Sci.* 114 (2018) 1846–1850. <https://www.currentscience.ac.in/Volumes/114/09/1846.pdf>.

Further reading

- [1] C₆₀.

Article

C₆₀ Fullerene Reduces the Level of Liver Damage in Chronic Alcohol Intoxication of Rats

Olexandr Motuziuk ^{1,2}, Dmytro Nozdrenko ¹, Svitlana Prylutska ³, Igor Vareniuk ¹ , Vsevolod Cherepanov ⁴ , Kateryna Bogutska ¹ , Sergii Rudenko ¹ , Yuriy Prylutsky ¹, Jacek Piosik ⁵  and Uwe Ritter ^{6,*} 

- ¹ ESC “Institute of Biology and Medicine”, Taras Shevchenko National University of Kyiv, Str. Volodymyrska 64/13, 01601 Kyiv, Ukraine; motuziuk.oleksandr@eenu.edu.ua (O.M.); dmytro.nozdrenko@knu.ua (D.N.); vareniukim@knu.ua (I.V.); bogutska_ki@knu.ua (K.B.); rudenkostrg@gmail.com (S.R.); prylut@ukr.net (Y.P.)
- ² Faculty of Biology and Forestry, Lesya Ukrainka Volyn National University, Av. Voli 13, 43025 Lutsk, Ukraine
- ³ Faculty of Plant Protection, Biotechnology and Ecology, National University of Life and Environmental Science of Ukraine, Str. Heroiv Oborony 13, 03041 Kyiv, Ukraine; psvit_1977@ukr.net
- ⁴ Institute of Physics, NAS of Ukraine, Av. Nauki 46, 03028 Kyiv, Ukraine; vvch2000@ukr.net
- ⁵ Intercollegiate Faculty of Biotechnology, University of Gdansk, Str. Abrahama 58, 80-307 Gdańsk, Poland; jacek.piosik@ug.edu.pl
- ⁶ Institute of Chemistry and Biotechnology, Technical University of Ilmenau, Str. Weimarer 23, 98693 Ilmenau, Germany
- * Correspondence: uwe.ritter@tu-ilmenau.de

Abstract: The liver is the main organ responsible for the metabolism of ethanol, which suffers significantly as a result of tissue damage due to oxidative stress. It is known that C₆₀ fullerenes are able to efficiently capture and inactivate reactive oxygen species in in vivo and in vitro systems. Therefore, the purpose of this study is to determine whether water-soluble C₆₀ fullerene reduces the level of pathological process development in the liver of rats induced by chronic alcohol intoxication for 3, 6, and 9 months, depending on the daily dose (oral administration; 0.5, 1, and 2 mg/kg) of C₆₀ fullerene throughout the experiment. In this context, the morphology of the C₆₀ fullerene nanoparticles in aqueous solution was studied using atomic force microscopy. Such biochemical parameters of experimental animal blood as ALT (alanine aminotransferase), AST (aspartate aminotransferase), GGT (gamma-glutamyl transferase) and ALP (alkaline phosphatase) enzyme activities, CDT (carbohydrate-deficient transferrin) level, values of pro-antioxidant balance indicators (concentrations of H₂O₂ (hydrogen peroxide) and GSH (reduced glutathione), activities of CAT (catalase), SOD (superoxide dismutase) and GP_x (selenium-dependent glutathione peroxidase)), and pathohistological and morphometric features of liver damage were analyzed. The most significant positive change in the studied biochemical parameters (up to 29 ± 2% relative to the control), as markers of liver damage, was recorded at the combined administration of alcohol (40% ethanol in drinking water) and water-soluble C₆₀ fullerenes in the optimal dose of 1 mg/kg, which was confirmed by small histopathological changes in the liver of rats. The obtained results prove the prospective use of C₆₀ fullerenes as powerful antioxidants for the mitigation of pathological conditions of the liver arising under prolonged alcohol intoxication.

Keywords: C₆₀ fullerene; liver; alcohol intoxication; biochemical blood parameters; histopathological analysis; atomic force microscopy



Citation: Motuziuk, O.; Nozdrenko, D.; Prylutska, S.; Vareniuk, I.; Cherepanov, V.; Bogutska, K.; Rudenko, S.; Prylutsky, Y.; Piosik, J.; Ritter, U. C₆₀ Fullerene Reduces the Level of Liver Damage in Chronic Alcohol Intoxication of Rats. *Molecules* **2024**, *29*, 2951. <https://doi.org/10.3390/molecules29132951>

Academic Editor: Adrian Keller

Received: 15 May 2024

Revised: 4 June 2024

Accepted: 20 June 2024

Published: 21 June 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The liver is the main organ responsible for the metabolism of ethanol, which suffers largely as a result of tissue damage due to oxidative stress. Alcohol-related liver diseases include fatty liver or steatosis, steatohepatitis, alcoholic hepatitis, and cirrhosis [1]. Alcohol metabolism also occurs in other organs, for example heart, brain, kidneys, pancreas, skeletal muscle, lungs, red blood cells, lymphocytes, etc. However, most studies on chronic

alcoholism point to the leading role of the liver in increasing oxidative stress and lipid peroxidation (LPO) [1]. Liver microsomes can oxidize ethanol in an NADH-dependent pathway catalyzed by iron [2,3]. The resulting acetaldehyde affects the immune system by altering the production of cytokines, antioxidant enzymes, and substances, especially mitochondrial and cytosolic glutathione. These compounds can cause toxic effects, as the proteins they bind to lose their functions and provoke an immune response [4].

Chronic alcohol consumption causes hypoxia in the pericentral liver, where additional oxygen is required for alcohol metabolism [5]. Hypoxia causes additional production of reactive oxygen species (ROSs) and LPO through the activity of xanthine oxyase in the liver [6]. In addition, a high alcohol concentration facilitates the absorption of endotoxins by altering intestinal permeability, modifying the intestinal microflora, and directly damaging the cells lining the inner gut [7]. Endotoxins physically attach to the surface of Kupffer cells via the CD14 receptor and Toll-like receptor 4 (TLR4) [8]. The results of study [9] indicate that the oxidative stress associated with chronic alcohol consumption can be explained by the activation of Kupffer cells by endotoxins.

The toxic effect of ethanol is in direct correlation with the concentration of acetaldehyde and acetate in the blood. During ethanol oxidation, there is an increased consumption of coenzyme NAD^+ , and an increase in the NADH/NAD^+ ratio, that plays an important role in the formation of fatty liver dystrophy. Acetaldehyde has a hepatotoxic effect, which is manifested due to the intensification of LPO processes, and the formation of compounds with other proteins and enzymes, which leads to the disruption of the function of phospholipid cell membranes. The complex of acetaldehyde compounds with proteins, including tubulin, causes changes in the structure of hepatocyte microtubules, forming the so-called alcoholic hyaline, and contributes to the disruption of intracellular transport, protein and water retention, and the development of balloon dystrophy of hepatocytes [10].

The current conventional diagnosis of alcoholic liver disease is based on a history of alcohol consumption and laboratory results of blood biochemical diagnostic parameters such as ALT (alanine aminotransferase), AST (aspartate aminotransferase), and GGT (gamma-glutamyl transferase) [11,12]. However, it is not possible to accurately determine the extent of liver damage while analyzing these biomarkers.

It is known that biocompatible and bioavailable C_{60} fullerene nanoparticles can efficiently capture and inactivate ROSs in in vivo and in vitro systems [13,14]. Recently it was demonstrated that applying water-soluble C_{60} fullerenes during chronic alcoholization of rats reduces the level of fluctuations of force muscle response (the so-called “tremor”) [15] and protects muscle tissue from damage caused by oxidative stress [16].

Thus, the purpose of this study was to determine whether water-soluble C_{60} fullerene reduces the level of pathological processes in the liver of rats caused by chronic alcohol intoxication, depending on the oral administration dose (“dose–effect”) together with alcohol throughout the experiment. In this context, the basic biochemical indices of experimental animal blood, as markers of liver damage, as well as the pathohistological and morphometric features of liver damage, were analyzed.

2. Results and Discussion

2.1. AFM Study

Atomic force microscopy (AFM) measurements showed that the C_{60} fullerene layer consisted of spatially separated randomly located point objects (Figure 1). The height of most of them did not exceed 2.1 nm. As can be seen in the Z-profile, the widths of the objects were in the range of 1.0–1.5 nm, which is close to the typical tip radius of the AFM probe used in the experiments. This allowed us to identify objects with a height of ~0.7 nm as individual C_{60} molecules, and higher objects as aggregates of molecules with a shape close to spherical. The size of most aggregates did not exceed three molecular diameters. Thus, according to the AFM data, C_{60} fullerene was in a molecular or low-aggregated state in the characterized solution. The isolated arrangement of C_{60} molecules (nanoclusters) can be explained by the existence of electrostatic repulsion forces between them. They

exhibited a high negative surface charge (zeta potential value was -25.3 mV at room temperature [17]), indicating a low tendency for aggregation in aqueous solution (i.e., a high solute stabilization).

Thus, the detected nanoformation of the C_{60} molecules in an aqueous solution (0.15 mg/mL) indicates their suitability for *in vivo* studies.

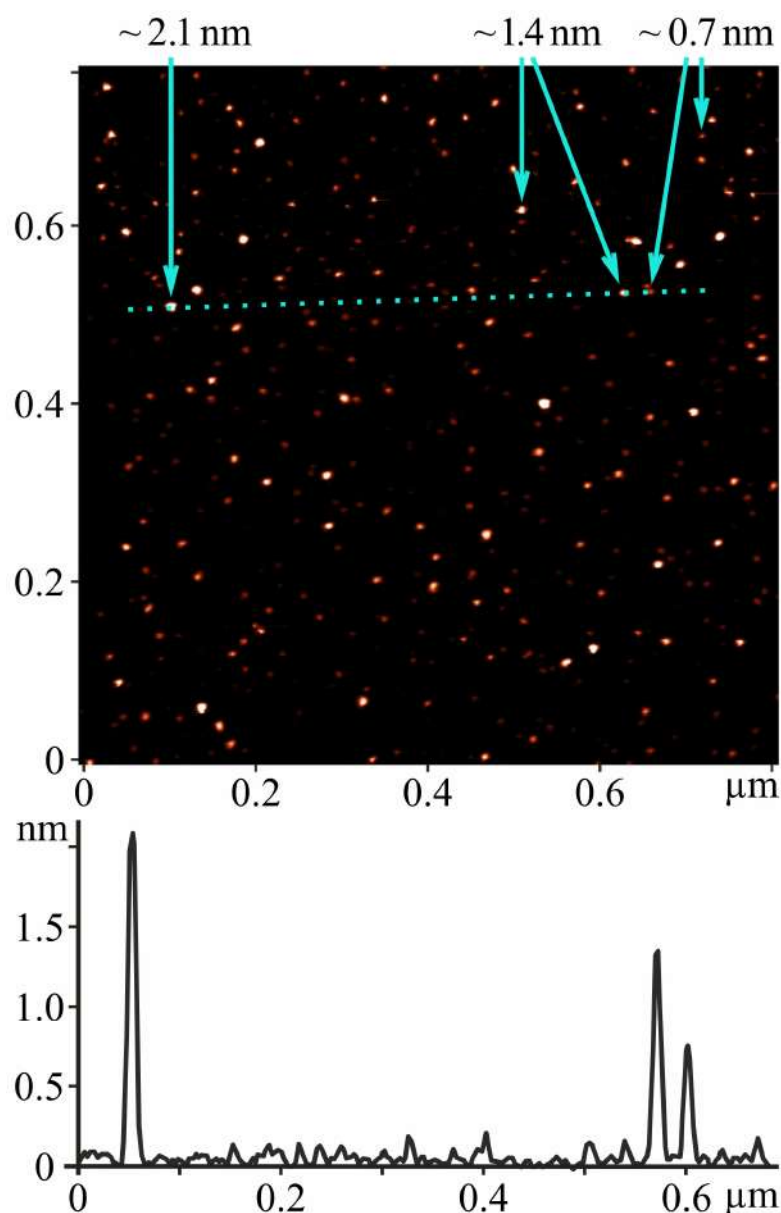


Figure 1. (Top): AFM image of single C_{60} molecules and their nanoaggregates deposited from C_{60} FAS (0.15 mg/mL) on a mica substrate. The arrows indicate the height of the nanoobjects (height ~ 0.7 nm corresponds to the diameter of the C_{60} molecule). (Bottom): Z-profile of the image along the marked dotted line.

2.2. BAC Value

The blood alcohol concentration (BAC) in animals after chronic ethanol consumption ranged from 142 ± 4 mg/dL (3 months of alcohol consumption) to 257 ± 5 mg/dL (9 months of alcohol consumption). These results agree well with the data [18]. C_{60} fullerene aqueous solution (C_{60} FAS) administration at a dose range of 0.5 , 1 , and 2 mg/kg together with ethanol did not lead to a reliable change in BAC values.

2.3. Biochemical Analysis

During chronic alcohol consumption, increased accumulation of LPO products, ROSs, glycosylated proteins, and carbohydrates leads to the formation of oxidative stress and may be the cause of cytolysis of hepatocytes, the main markers of which are the liver enzymes ALT, AST, and ALP (alkaline phosphatase) [19].

ALT is an enzyme whose highest amount is produced by the liver, heart muscle, pancreas, kidney, and muscle cells, so its increase in blood indicates damage to these organs [20].

ALT activity increased from 31 ± 1 Units/L in the norm (intact group) to 56 ± 2 , 67 ± 3 , and 74 ± 3 Units/L during alcoholization lasting 3, 6, and 9 months (control), respectively (Figure 2). The co-administration of ethanol and C₆₀FAS (dose 1 mg/kg) decreased its activity by $11 \pm 1\%$, $16 \pm 1\%$, and $18 \pm 1\%$ during alcoholization of 3, 6, and 9 months, respectively, relative to the control. This index did not significantly change with an increase in the dose of C₆₀FAS to 2 mg/kg, and reducing the dose of C₆₀FAS to 0.5 mg/kg decreased its positive effect by $11 \pm 1\%$ relative to the group “alcoholization+C₆₀ (1 mg/kg)”.

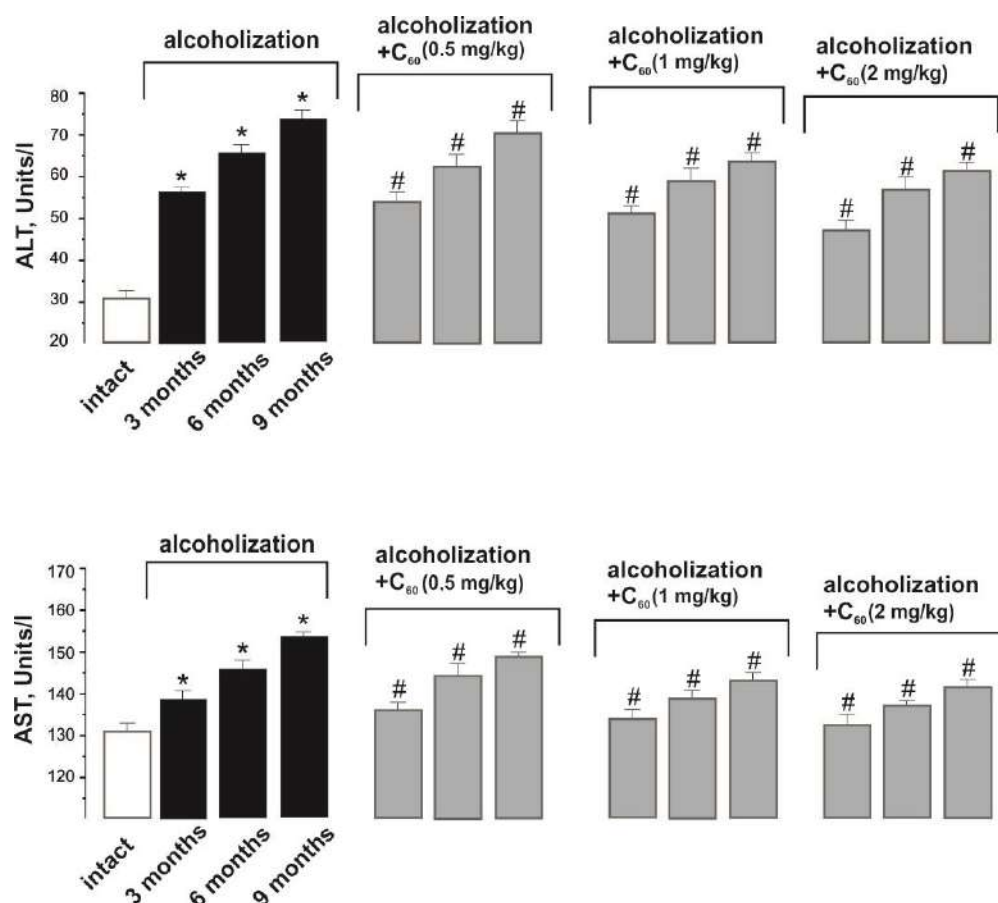


Figure 2. Activities of AST and ALT in the blood of alcoholized animals: intact—rats receiving 100% drinking water; alcoholization—alcoholized rats (control); alcoholization+C₆₀ (0.5, 1 and 2 mg/kg)—rats receiving a mixture of ethanol and C₆₀FAS at doses of 0.5, 1, and 2 mg/kg, respectively, throughout alcoholization; 3, 6, and 9 months—alcoholization of 3, 6, and 9 months, respectively. * $p < 0.05$ relative to the intact group; # $p < 0.05$ relative to the alcoholization group.

AST is an enzyme, the largest amount of which is produced by liver and heart cells in pathology. Normally only a small part of this enzyme is present in the blood [20].

The increase in AST activity from 132 ± 1 Units/L in the norm (intact group) to 138 ± 6 , 142 ± 7 , and 154 ± 6 Units/L during alcoholization lasting 3, 6, and 9 months (control), respectively, is a consequence of progressive destruction of hepatocytes (Figure 2). The

co-administration of ethanol and C₆₀FAS (dose 1 mg/kg) decreased its activity by $10 \pm 1\%$ during the experiment relative to the control. This index did not significantly change with an increase in the dose of C₆₀FAS to 2 mg/kg, and reducing the dose of C₆₀FAS to 0.5 mg/kg decreased its positive effect by $10 \pm 1\%$ relative to the group “alcoholization+C₆₀ (1 mg/kg)”.

In clinical practice, the ratio of AST to ALT activity in the blood (de Ritis ratio = AST/ALT) is used to diagnose liver diseases [20]. As mentioned above, these enzymes have organ specificity; in particular, in case of liver damage due to alcohol intoxication, increased activity of ALT in blood is detected.

The decrease in the de Ritis ratio from 0.97 ± 0.05 in the norm (intact group) to 0.80 ± 0.07 , 0.68 ± 0.07 , and 0.78 ± 0.07 at 3, 6, and 9 months of alcoholization indicates progressive disturbance of hepatocyte functioning, which is in good agreement with the data [20]: at the initial stage of alcohol intoxication, the de Ritis ratio was equal to 0.6. The co-administration of ethanol and C₆₀FAS (dose 1 mg/kg) increased the de Ritis ratio by $14 \pm 1\%$ during the experiment relative to the control (ALT activity decreases more strongly than AST activity).

GGT is an enzyme, the largest amount of which is accumulated in the liver, kidney, and pancreatic cells. It takes part in the metabolism of amino acids and synthesizing protein molecules. Unlike other liver enzymes, GGT production is “triggered” by alcohol, so its activity may be increased in abusers even in the absence of liver disease. This enzyme is more sensitive to disturbances in liver cells than ALT and AST [21].

The increase in GGT activity from 1.6 ± 0.1 Units/L in the norm (intact group) to 3.4 ± 0.1 , 4.4 ± 0.1 , and 5.9 ± 0.2 Units/L during alcoholization lasting 3, 6, and 9 months (control), respectively, is an indicator of progressive liver cell destruction (Figure 3). The co-administration of ethanol and C₆₀FAS (dose 1 mg/kg) reduced the described indices by $15 \pm 1\%$, $18 \pm 1\%$, and $22 \pm 1\%$ during alcoholization of 3, 6, and 9 months, respectively, relative to the control. This index did not significantly change with an increase in the dose of C₆₀FAS to 2 mg/kg, and decreasing the dose of C₆₀FAS to 0.5 mg/kg reduced its positive effect by $10 \pm 1\%$ relative to the group “alcoholization+C₆₀ (1 mg/kg)”.

ALP is an enzyme that catalyzes the removal of phosphate groups from organic compounds, such as proteins and nucleotides, and is involved in the transport of phosphorus and calcium in the body. ALP activity in the blood increases as a result of liver diseases, particularly hepatitis caused by alcohol intoxication [22].

ALP activity increased from 8.8 ± 0.5 Units/L in the norm (intact group) to 9.4 ± 0.7 , 9.8 ± 0.7 , and 10.3 ± 0.7 Units/L during alcoholization lasting 3, 6, and 9 months (control), respectively (Figure 3). The co-administration of ethanol and C₆₀FAS (dose 1 mg/kg) decreased ALP activity by $10 \pm 1\%$ during the experiment relative to the control. This index did not significantly change with an increase in the dose of C₆₀FAS to 2 mg/kg, and reducing the dose of C₆₀FAS to 0.5 mg/kg decreased its positive effect by $10 \pm 1\%$ relative to the group “alcoholization+C₆₀ (1 mg/kg)”.

Transferrin is a blood plasma protein that transports iron ions. The liver is the main source of transferrin production, the main role of which is to deliver iron from absorption centers in the duodenum to the rest of the tissues and digestion of red blood cells by macrophages. The CDT (carbohydrate-deficient transferrin) level is an important biomarker of chronic alcohol intoxication. The advantage of using this index over others is that it is more specific and rarely produces false-positive reactions in liver damage of alcoholic etiology [23].

The CDT level increased from $1.54 \pm 0.10\%$ in the norm (intact group) to $1.65 \pm 0.1\%$, $1.75 \pm 0.1\%$, and $1.94 \pm 0.1\%$ during alcoholization lasting 3, 6, and 9 months (control), respectively (Figure 3). The co-administration of ethanol and C₆₀FAS (dose 1 mg/kg) decreased CDT levels by $10 \pm 1\%$ during the experiment relative to the control. This index did not significantly change with an increase in the dose of C₆₀FAS to 2 mg/kg, and decreasing the dose of C₆₀FAS to 0.5 mg/kg reduced its positive effect by $10 \pm 1\%$ relative to the “alcoholization+C₆₀ (1 mg/kg)” group.

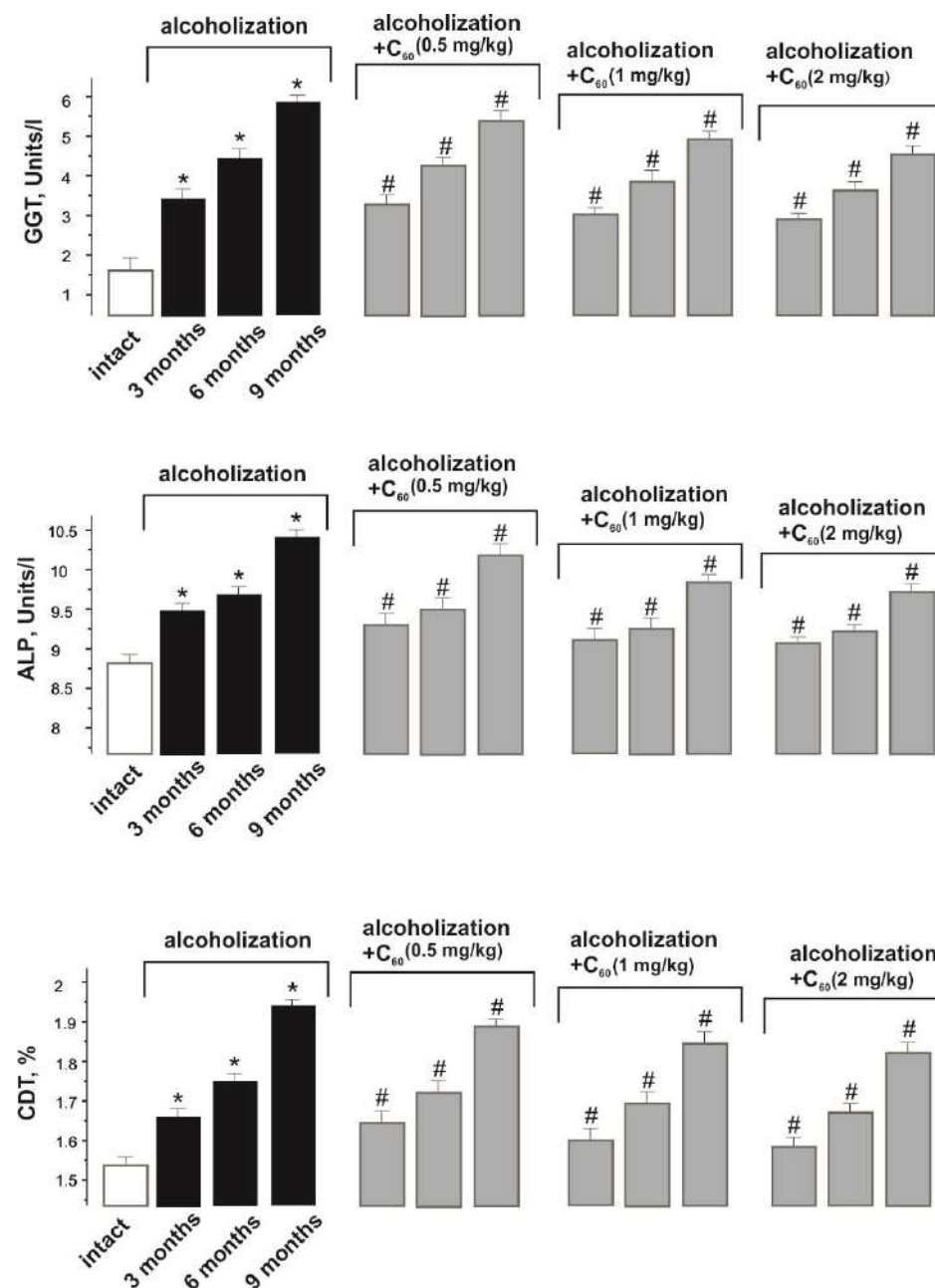


Figure 3. GGT and ALP activities and CDT level in the blood of alcoholized animals: intact—rats receiving 100% drinking water; alcoholization—alcoholized rats (control); alcoholization+C₆₀ (0.5, 1, and 2 mg/kg)—rats receiving a mixture of ethanol and C₆₀FAS at doses of 0.5, 1, and 2 mg/kg, respectively, throughout alcoholization; 3, 6, and 9 months—alcoholization of 3, 6, and 9 months, respectively. * $p < 0.05$ relative to the intact group; # $p < 0.05$ relative to the alcoholization group.

The effects of C₆₀ fullerenes on the in vivo models [19,20] can be explained by their antioxidant properties. To confirm this hypothesis in our case, we assessed the indicators of pro-/antioxidant balance in the blood of the studied rats.

The data presented in Figure 4 indicate an increased level of LPO and oxidative stress in the blood of rats after prolonged alcohol intoxication. Thus, H₂O₂ (hydrogen peroxide) concentration increased by $151 \pm 9\%$, $327 \pm 12\%$, and $414 \pm 17\%$ during alcoholization of 3, 6, and 9 months, respectively, compared with the intact group. The co-administration of ethanol and C₆₀FAS (dose 1 mg/kg) decreased H₂O₂ content by $24 \pm 2\%$, $27 \pm 2\%$, and $20 \pm 2\%$ during alcoholization lasting 3, 6, and 9 months, respectively, relative to the control. These data confirm that a water-soluble C₆₀ fullerene can penetrate through the

cell membranes without signs of their damage [21]. Finally, this index did not significantly change with an increase in the dose of C₆₀FAS to 2 mg/kg, and decreasing the dose of C₆₀FAS to 0.5 mg/kg reduced its positive effect by 14–16 ± 1% relative to the group “alcoholization+C₆₀ (1 mg/kg)”.

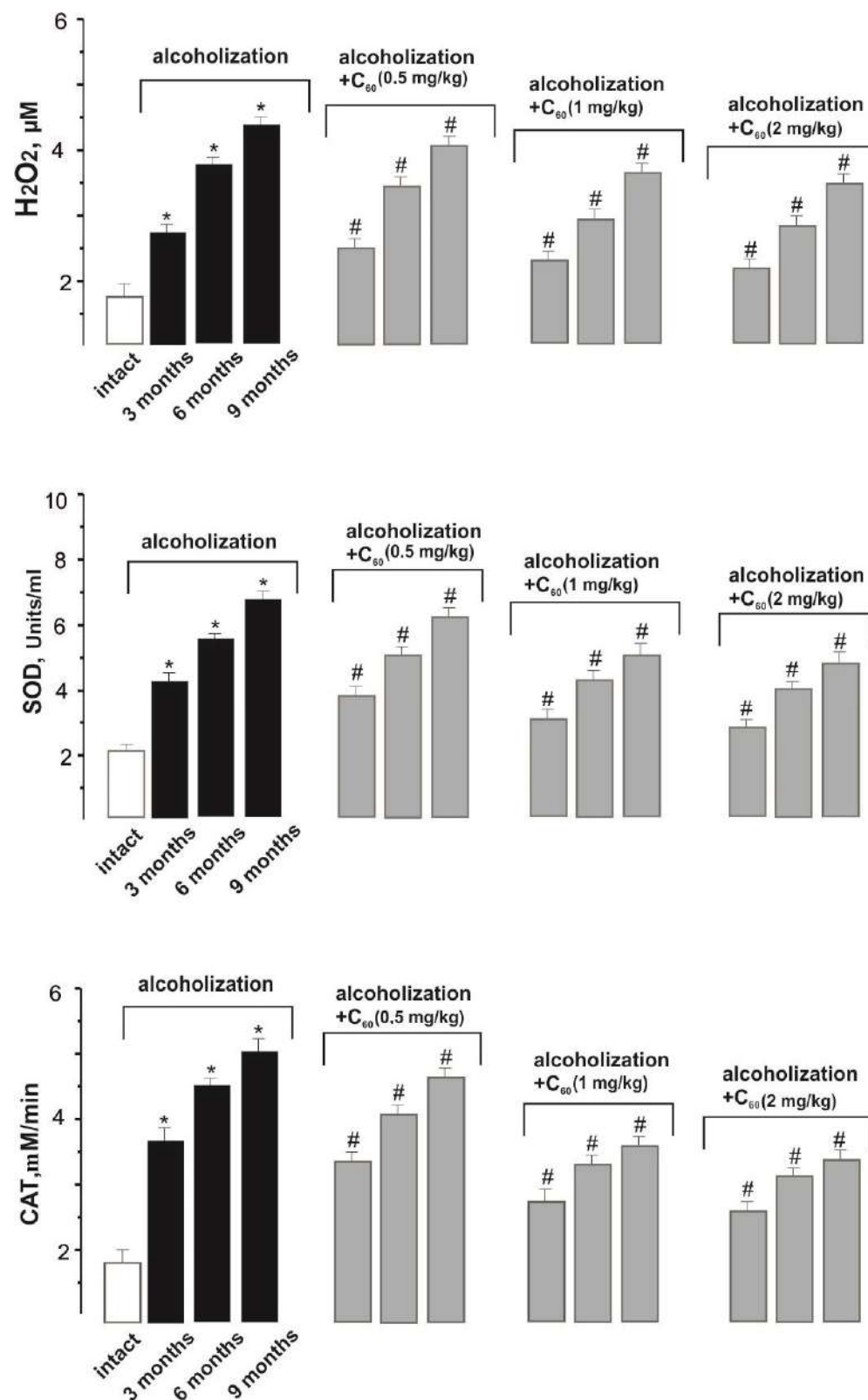


Figure 4. Cont.

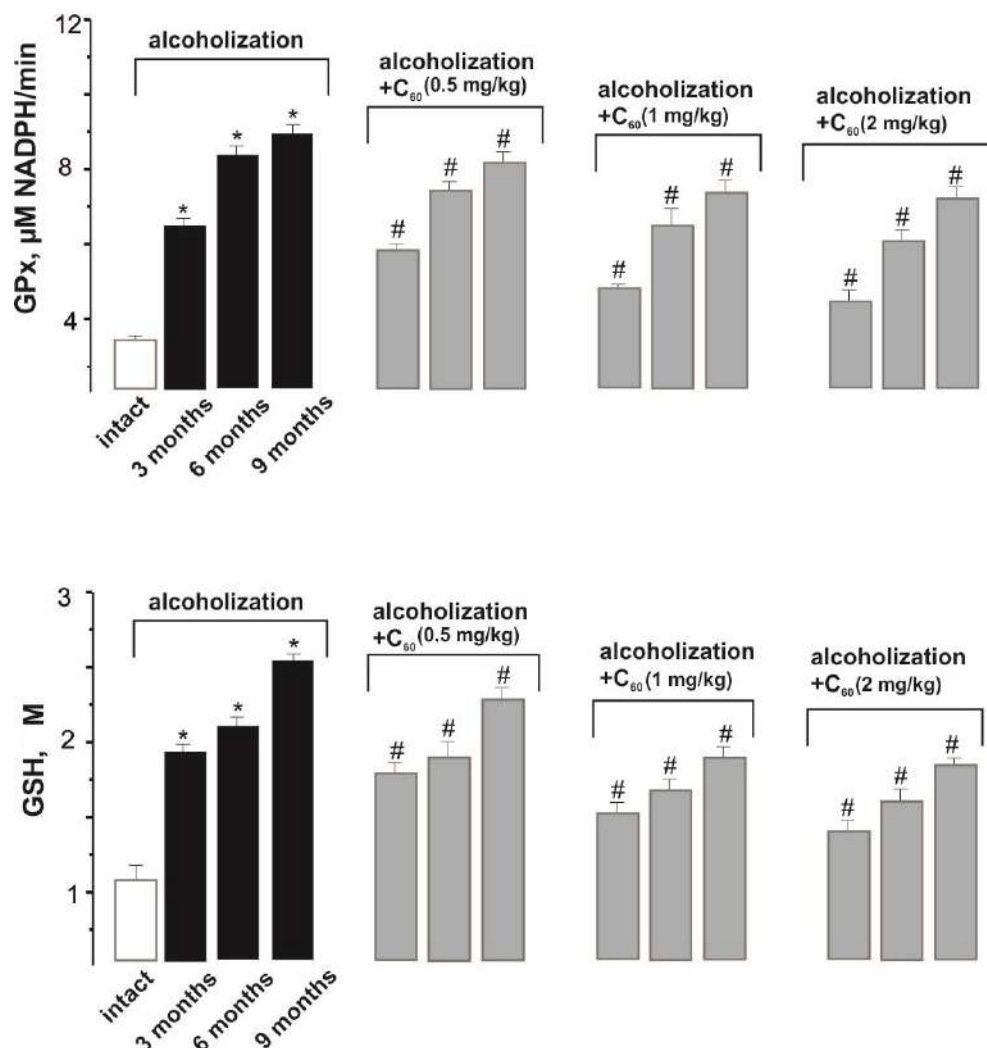


Figure 4. Pro- and antioxidant balance indicators (H_2O_2 , SOD, CAT, GP_x , and GSH) in the blood of alcoholized animals: intact—rats receiving 100% drinking water; alcoholization—alcoholized rats (control); alcoholization+C₆₀ (0.5, 1, and 2 mg/kg)—rats receiving a mixture of ethanol and C₆₀FAS at doses of 0.5, 1, and 2 mg/kg, respectively, throughout alcoholization; 3, 6 and 9 months—alcoholization of 3, 6, and 9 months, respectively. * $p < 0.05$ relative to the intact group; # $p < 0.05$ relative to the alcoholization group.

A key role in the mechanisms of regulation of free radical and peroxide processes in the body belongs to such antioxidant defense enzymes as SOD (superoxide dismutase), CAT (catalase), GP_x (selenium-dependent glutathione peroxidase), and GSH (reduced glutathione). SOD is the most powerful natural antioxidant and enzyme of the first link of antioxidant defense, which performs the dismutation reaction of superoxide anion radicals and transforms them into less reactive hydrogen peroxide molecules [24].

In the experiment against the background of combined use of ethanol and C₆₀FAS (dose 1 mg/kg), a gradual decrease in SOD activity by $25 \pm 2\%$, $29 \pm 2\%$, and $30 \pm 2\%$ was found during alcoholization of 3, 6, and 9 months, respectively, relative to the control (Figure 4). This index did not significantly change with an increase in the dose of C₆₀FAS to 2 mg/kg, and reducing the dose of C₆₀FAS to 0.5 mg/kg decreased its positive effect by $15\text{--}18 \pm 1\%$ relative to the group “alcoholization+C₆₀ (1 mg/kg)”.

CAT activity was decreased by $27 \pm 2\%$, $29 \pm 2\%$, and $30 \pm 2\%$ when ethanol and C₆₀FAS were co-administered at a dose of 1 mg/kg during alcoholization of 3, 6, and 9 months, respectively, relative to the control (Figure 4). This index did not significantly change with an increase in the dose of C₆₀FAS to 2 mg/kg, and decreasing the

dose of C₆₀FAS to 0.5 mg/kg reduced its positive effect by $14\text{--}19 \pm 1\%$ relative to the “alcoholization+C₆₀ (1 mg/kg)” group.

The cellular mechanisms of the body’s antioxidant defense are also associated with a powerful glutathione link functioning [24]. The glutathione system is one of the active components of antioxidant defense along with antiradical enzymes, which not only prevents the course of free radical reactions but also ensures the effective elimination of the final metabolites of LPO [25].

In the experiment against the background of combined use of ethanol and C₆₀FAS (dose 1 mg/kg), a decrease in GP_x activity by $24 \pm 2\%$, $25 \pm 2\%$, and $20 \pm 2\%$ was found during alcoholization of 3, 6, and 9 months, respectively, relative to the control (Figure 4). This index did not significantly change with an increase in the dose of C₆₀FAS to 2 mg/kg, and decreasing the dose of C₆₀FAS to 0.5 mg/kg reduced its positive effect by $11\text{--}17 \pm 1\%$ relative to the group “alcoholization+C₆₀ (1 mg/kg)”.

The co-administration of ethanol and C₆₀FAS (dose 1 mg/kg) decreased GSH level by $21 \pm 2\%$, $27 \pm 2\%$, and $25 \pm 2\%$ during alcoholization lasting 3, 6, and 9 months, respectively, relative to the control (Figure 4). This agrees well with preliminary studies [26], where it was shown that C₆₀ fullerenes affect the processes of glutathione synthesis and metabolism in different tissues under various pathologies by modulating the Nrf2/ARE-antioxidant pathway. This index did not significantly change with an increase in the dose of C₆₀FAS up to 2 mg/kg, and decreasing the dose of C₆₀FAS up to 0.5 mg/kg reduced its positive effect by $11\text{--}17 \pm 1\%$ relative to the “alcoholization+C₆₀ (1 mg/kg)” group.

Summarizing the above biochemical data, we can conclude that 1 mg/kg is the optimal dose of C₆₀FAS administration: reducing it by half significantly decreases the therapeutic effect of C₆₀ fullerenes, and doubling it does not lead to a significant increase in the observed effects.

2.4. Histological Analysis

In intact rats, no histopathological features were observed. Only a small number of pyknotic nuclei in hepatocytes were present (Figure 5A).

In the liver of alcoholized rats, within 9 months (longest experiment duration), there is a strong hydropic dystrophy of hepatocytes with an increase in cell size (as a result of edema or as a result of increased functional load on hepatocytes) (Table 1; Figure 5B). Some of the cell nuclei are pyknotic, others are enlarged and bright. Also, six animals have small-droplet lipid dystrophy, and four animals have strong large-droplet lipid dystrophy and balloon dystrophy of hepatocytes (Figure 5B). Necrotic hepatocytes are found, in great number, in 5 of 10 rats. Some of the blood vessels are dilated, with leukocyte infiltrates around. There is a weak expansion of connective tissue.

In the liver of alcoholized rats that received C₆₀FAS (optimal dose 1 mg/kg), within 9 months, moderately expressed hydropic and weakly expressed small-droplet lipid dystrophy of hepatocytes are observed, and balloon dystrophy is absent (Figure 5C). The sizes of the cells and the volumes of their nuclei are increased (Table 1). Hepatocytes with pyknotic nuclei and necrotic hepatocytes are present but in significantly lower numbers than in alcoholized animals that did not receive C₆₀FAS. Occasionally there are dilated blood vessels, with leukocyte infiltrates around. There is a weak expansion of connective tissue (Figure 5C). Therefore, histopathological changes in the liver of rats that received C₆₀FAS together with alcohol are clearly smaller.

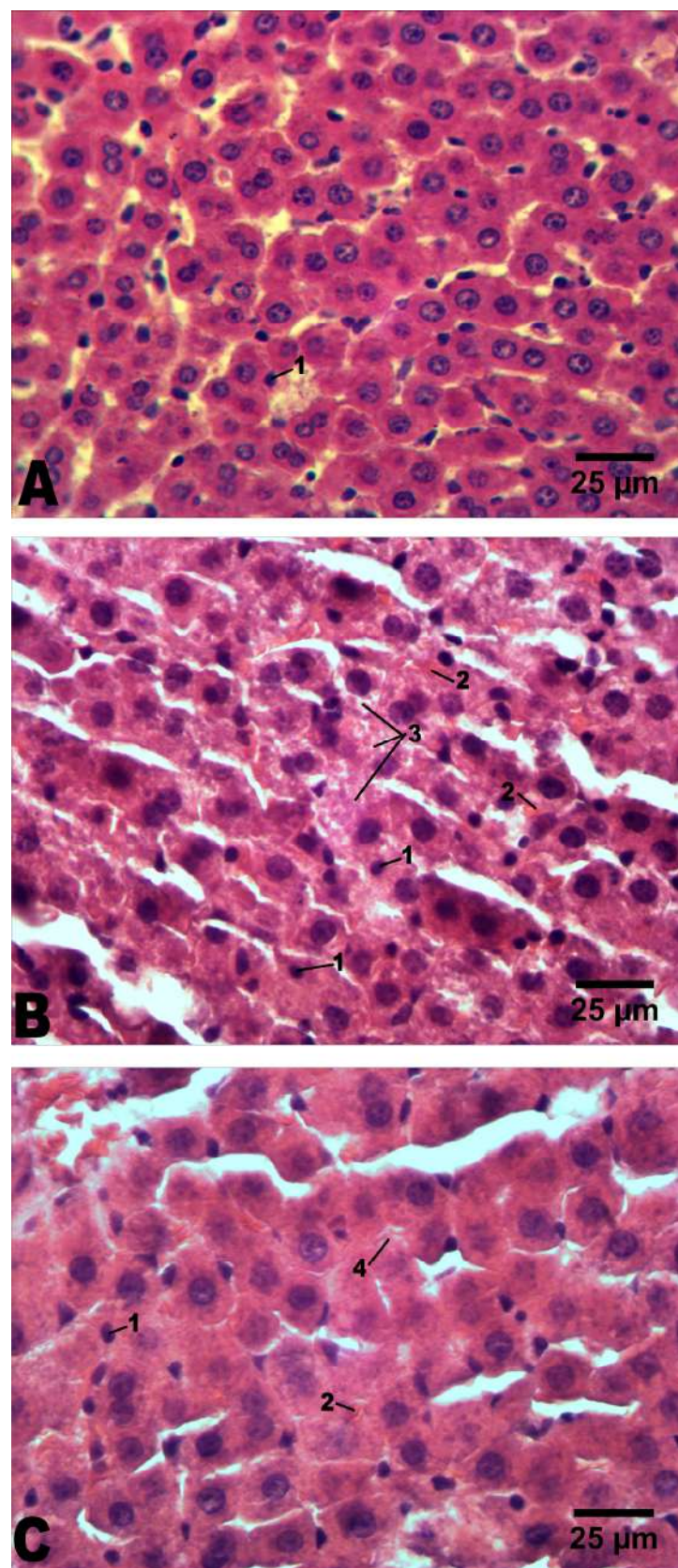


Figure 5. Representative histological images of the liver: (A)—intact group; (B)—alcoholization group; (C)—alcoholization+C₆₀ (1 mg/kg) group. H&E (hematoxylin and eosin) staining. Scale bar: 25 µm. 1—pyknotic nuclei; 2—Mallory bodies; 3—small-droplet lipid and balloon dystrophy in alcoholization group; 4—hydropic dystrophy in alcoholization+C₆₀ (1 mg/kg) group.

Table 1. Histopathological features of the liver in rats after alcohol and C₆₀FAS consumption within 9 months.

Histopathological Features	Intact	Alcoholization	Alcoholization+C ₆₀ (1 mg/kg)
Volume of hepatocyte's nucleus, μm^3	138 $\pm$ 8	248 $\pm$ 11 *	284 $\pm$ 19 #
Cross-sectional area of hepatocyte, μm^2	205 $\pm$ 11	273 $\pm$ 20 *	244 $\pm$ 11 #
Pyknotic nuclei in hepatocytes	+	++	+
Mallory bodies	–	+++	++
Lipid dystrophy of hepatocytes	–	+++	+
Hydropic dystrophy of hepatocytes	–	+++	++
Balloon dystrophy of hepatocytes	–	++	–
Necrotic hepatocytes	–	++	+
Blood vessel dilatation	–	++	+
Inflammatory cell infiltration	–	++	++
Expansion of connective tissue (fibrosis)	–	+	+

Trait intensity: “–” —not observed; “+” —weak; “++” —moderate; “+++” —strong. * $p < 0.05$ compared with the intact group; # $p < 0.05$ compared with the alcoholization group.

The authors [27] showed that more than 90% of alcohol consumed is metabolized through oxidative and non-oxidative pathways with the formation of such reactive compounds as acetate, acetaldehyde, fatty acid ethyl ester, etc. These compounds generate ROSs, which causes an increase in oxidative stress and LPO, thus disrupting the structural integrity of cells and, accordingly, the functions of tissues and organs. In our opinion, a potential mechanism of the C₆₀ fullerene effects described above are related to their powerful antioxidant properties [13,14]: by effectively inactivating ROSs, C₆₀ fullerenes reduce the number of damaged hepatocytes and thus reduce the severity of alcohol intoxication. The results obtained here are in good agreement with the previous data on the C₆₀ fullerene inhibition of liver inflammation and improvement in liver and pancreas state under acute and chronic cholangitis in rats [28]. Moreover, the results of [29,30] indicate an indirect effect of antioxidants on the increase in LPO indicators in alcohol-induced muscle damage. It has been shown that the use of N-acetylcysteine can attenuate the toxic effects of alcohol [31]. A study [32] demonstrated that procysteine use increases mitochondrial glutathione levels and thereby alleviates alcohol-induced oxidative stress.

3. Materials and Methods

3.1. Preparation and Characterization of C₆₀FAS

A unique method based on the transfer of C₆₀ fullerenes from toluene into water with simultaneous ultrasonic treatment (8 Hz, 8 h) was used to prepare the highly stable reproducible C₆₀FAS [33]. The resulting dark brown C₆₀FAS (maximum concentration 0.15 mg/mL) is stable for 12–18 months in the temperature range of +4–25 °C.

The structural state of C₆₀ fullerene was studied using AFM after deposition of its layer from a drop of C₆₀FAS on a mica substrate and evaporation of water. AFM measurement was performed using the “Solver Pro M” (NT-MDT) system in the amplitude modulation tapping mode using probes “RTESPA-150” (Bruker, Preston, VIC, Australia, ~150 kHz, 5–10 N/m, $R_{\text{tip}} < 12$ nm). The free oscillations’ amplitude of the cantilever was set to 50 nm. No image processing techniques other than surface tilt subtraction were used.

3.2. In Vivo Experiments

The experiments were carried out on male Wistar rats aged 1 to 10 months (at the end of the experiment). The initial weight of the animals was 170 ± 10 g.

The study protocol was approved by the Bioethics Committee of the ESC “Institute of Biology and Medicine” of Taras Shevchenko National University of Kyiv (protocol No. 2 dated 2 September 2022) in accordance with the rules of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and the norms of biomedical ethics in accordance with the Law of Ukraine No. 3447—IV of 21 February 2006, Kyiv, “On the Protection of Animals from Cruelty”, as well as the European Union Directive (2010/63/EU) “On the protection of animals used for scientific purposes”.

During the experiment, we used the following groups of animals:

- The intact group of rats ($n = 10$) received 100% drinking water;
- Rats in the “alcoholization” group (control; $n = 30$; 10 animals in each group) were randomly selected. Each rat was placed in a separate cage to receive 40% ethanol in drinking water [34], which meant that the animals did not have access to 100% water until they had consumed the dosed portion of ethanol [18]. The amount of ethanol consumed was calculated with respect to 0.5% of the animal’s body weight. Recalculation of the ethanol dose was performed every 24 h throughout the experiment [35]. The duration of alcoholization was 3, 6, and 9 months. The target value of 40% ethanol in drinking water was chosen because it reflects the blood alcohol concentrations reported in chronic alcoholics [32];
- Rats of the “alcoholization+C₆₀” groups ($n = 90$; 10 animals in each group) together with ethanol were orally administered C₆₀FAS at daily doses of 0.5, 1, and 2 mg/kg of rat weight. Recalculation of the C₆₀FAS dose was performed every 24 h throughout the experiment. Control of the amount of C₆₀FAS ingested was performed by denying the animals access to drinking 100% water until they fully consumed the applied drug.

Note that the tested doses of C₆₀FAS are significantly lower than the LD₅₀ value, which was 600 mg/kg body weight when administered orally to rats [36] and 721 mg/kg when administered intraperitoneally to mice [17]. It has been established that after intravenous administration to mice, C₆₀ fullerenes accumulate predominantly in the blood, spleen, stomach, and liver and are excreted from the body within 72 h, mainly with urine [37].

Finally, it should also be emphasized that in our recent research [38], various regimens of C₆₀FAS administration (1 mg/kg) were studied during rat alcoholization lasting 3, 6, and 9 months, namely, 1 h before drinking alcohol, together with alcohol, and 1 h after drinking alcohol. Based on the assessment of the biochemical indices of blood and tissue of the *muscle gastrocnemius* chronically alcoholized rats, the most optimal regimen for administering C₆₀FAS was determined, namely, together with alcohol, the positive effects of which exceeded other regimens for the administration of this pharmacological agent.

Animals in the study groups were euthanized (carbon dioxide-induced hypoxia) on the last day of the experiment, and all efforts were made to minimize suffering. During the study, animals were observed daily for visual clinical signs according to the FELASA recommendations [39].

3.3. Biochemical Analysis

The activities of the enzymes ALT, AST, GGT, and ALP, the level of CDT, and the values of pro-antioxidant balance indicators (H₂O₂ and GSH contents, CAT, SOD, and GP_x activities) in the blood of rats as markers of liver dysfunction were determined using clinical diagnostic equipment—biochemical analyzers RNL-200 (The Netherlands) and ABX Micros ESV60 (France).

The BAC value in rats was measured at the end of experiment using an AM1 alcohol analyzer (Analox Instruments Limited, Stourbridge, UK).

3.4. Histological Analysis

The liver samples were separated and fixed in 10% formalin. They were then embedded in paraffin, cut into 5 µm thick sections, and stained with H&E [40]. Digital micrographs of stained sections were taken at a magnification of ×400 by using a computer-assisted

image analyzing system (Olympus BX41 microscope and Olympus C-5050 Zoom digital camera). The histopathological profiles of the liver were determined by light microscopy observation. Intensity of each histopathological feature was evaluated as follows: “—” —not observed; “+” —slight; “++” —moderate; “+++” —strong. Diameter of hepatocytes’ nuclei and cross-sectional area of hepatocytes were measured using ImageJ software [41]. Volume of hepatocytes’ nuclei was calculated using formula $V = \frac{\pi}{6} D^2$, where D is the diameter of hepatocytes’ nuclei.

3.5. Statistics

The experimental results were processed by methods of variation statistics using the Statistica 13.3 program. At least three repeats were performed for each measurement. A *t*-test (for one experimental group) or one-way ANOVA (for three experimental groups) followed by a post hoc Dunnett multiple comparison test to compare all data vs. control were used. A factorial ANOVA test was also used depending on such factors of variation as dose and treatment period. Assumptions that the data followed a normal distribution and had identical standard deviations were tested using the Shapiro–Wilk and Bartlett methods, respectively. The differences among experimental groups were considered statistically significant at $p < 0.05$.

4. Conclusions

Thus, in all tests performed after chronic alcoholization during 3, 6, and 9 months, when alcohol and C₆₀FAS at the optimal dose of 1 mg/kg were co-administered, there was a positive change in the studied biochemical parameters of rat blood, as markers of liver damage, up to $29 \pm 2\%$ relative to the control. Ultimately, the above biochemical results were confirmed by histopathological data. It can be concluded that the use of C₆₀FAS contributes to the reduction in oxidative processes in tissues by maintaining a balance between pro-oxidants and the body’s antioxidant defense system. This prevents the negative impact of ROSs on cellular and subcellular structures during prolonged alcohol intoxication. On this basis, it can be assumed that C₆₀ fullerenes, as powerful nanoantioxidants, are able to correct pathological conditions of the liver arising in chronic alcohol intoxication, which requires further clinical trials.

Author Contributions: Conceptualization, supervision, Y.P.; methodology, O.M. and D.N.; investigation, O.M., D.N., S.P., K.B., I.V., V.C. and U.R.; formal analysis, S.R.; project administration, J.P.; writing—original draft preparation, D.N.; writing—review and editing, Y.P., J.P. and U.R.; funding acquisition, U.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study protocol was approved by the Bioethics Committee of the ESC “Institute of Biology and Medicine” of Taras Shevchenko National University of Kyiv (protocol No. 2 dated 2 September 2022) in accordance with the rules of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and the norms of biomedical ethics in accordance with the Law of Ukraine No. 3447—IV of 21 February 2006, Kyiv, “On the Protection of Animals from Cruelty”, as well as the European Union Directive (2010/63/EU) “On the protection of animals used for scientific purposes”.

Informed Consent Statement: Not applicable.

Data Availability Statement: Dataset available upon request from the authors.

Acknowledgments: We acknowledge support for the publication costs by the Open Access Publication Fund of the Technical University of Ilmenau, Germany.

Conflicts of Interest: The authors declare no conflicts of interest.

References

- Liu, S.-Y.; Tsai, I.-T.; Hsu, Y.-C. Alcohol-Related Liver Disease: Basic Mechanisms and Clinical Perspectives. *Int. J. Mol. Sci.* **2021**, *22*, 5170. [\[CrossRef\]](#) [\[PubMed\]](#)
- Pérez, M.J.S.; González-Reimers, E.; Santolaria-Fernández, F.; de la Vega-Prieto, M.J.; Martínez-Riera, A.; González, P.A.; Rodríguez, E.R.; Durán-Castellón, M.C. Lipid peroxidation and serum cytokines in acute alcoholic hepatitis. *Alcohol. Alcohol.* **2006**, *41*, 593–597. [\[CrossRef\]](#) [\[PubMed\]](#)
- Dey, A.; Cederbaum, A.I. Alcohol and oxidative liver injury. *Hepatology* **2006**, *43*, S63–S74. [\[CrossRef\]](#) [\[PubMed\]](#)
- Kashinakunti, S.V.; Kallaganada, G.S.; Rangappa, M.; Hiremath, K.; Bannale, S. Are we over estimating serum oxidative stress/lipid peroxidation in alcoholic liver diseases? A review. *Free. Radic. Antioxid.* **2012**, *2*, 1–9. [\[CrossRef\]](#)
- Manzo-Aralos, S.; Saavedra-Molina, A. Cellular and mitochondrial effects of alcohol consumption. *Int. J. Environ. Res. Public Health* **2010**, *7*, 4281–4304. [\[CrossRef\]](#) [\[PubMed\]](#)
- El-Sokkary, G.H.; Khidr, B.M.; Younes, H.A. Role of melatonin in reducing hypoxia-induced oxidative stress and morphological changes in the liver of male mice. *Eur. J. Pharmacol.* **2006**, *50*, 107–114. [\[CrossRef\]](#) [\[PubMed\]](#)
- Hines, I.N.; Wheeler, M.D. Recent advances in alcoholic liver disease III. Role of the innate immune response in alcoholic hepatitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2004**, *287*, G310–G314. [\[CrossRef\]](#) [\[PubMed\]](#)
- Wheeler, M.D. Endotoxin and Kupffer Cell Activations in Alcoholic Liver Disease. *Alcohol. Res. Health* **2003**, *27*, 300–306. [\[PubMed\]](#)
- Aksoy, H.; Koruk, M.; Akcay, F. The Relationship Between Serum Malondialdehyde and Ceruloplasmin in Chronic Liver Disease. *Turk. J. Biochem.* **2003**, *28*, 32–34.
- Yang, L.; Latchoumycandane, C.; McMullen, M.R.; Pratt, B.T.; Zhang, R.; Papouchado, B.G.; Nagy, L.E.; Feldstein, A.E.; McIntyre, T.M. Chronic alcohol exposure increases circulating bioactive oxidised phospholipids. *J. Biol. Chem.* **2010**, *285*, 22211–22220. [\[CrossRef\]](#)
- Hong-Brown, L.Q.; Frost, R.A.; Lang, C.H. Alcohol impairs protein synthesis and degradation in cultured skeletal muscle cells. *Alcohol. Clin. Exp. Res.* **2001**, *25*, 1373–1382. [\[CrossRef\]](#) [\[PubMed\]](#)
- Subramanian, V.; Chakravarthi, S.; Jegasothy, R.; Seng, W.Y.; Fuloria, N.K.; Fuloria, S.; Hazarika, I.; Das, A. Alcohol-associated liver disease: A review on its pathophysiology, diagnosis and drug therapy. *Toxicol. Rep.* **2021**, *8*, 376–385. [\[CrossRef\]](#) [\[PubMed\]](#)
- Eswaran, S.V. Water Soluble Nanocarbon Materials: A Panacea for All? *Curr. Sci.* **2018**, *114*, 1846–1850. [\[CrossRef\]](#)
- Ferreira, C.A.; Ni, D.; Rosenkrans, Z.T.; Cai, W. Scavenging of reactive oxygen and nitrogen species with nanomaterials. *Nano Res.* **2018**, *11*, 4955–4984. [\[CrossRef\]](#) [\[PubMed\]](#)
- Motuziuk, O.; Nozdrenko, D.; Prylutska, S.; Bogutska, K.; Mishchenko, I.; Abramchuk, O.; Khrapatyi, S.; Ritter, U.; Prylutsky, Y. C₆₀ fullerene reduces the level of fluctuations in the force response of *muscle gastrocnemius* in chronically alcoholized rats. *Appl. Nanosci.* **2023**, *13*, 7057–7067. [\[CrossRef\]](#)
- Motuziuk, O.; Nozdrenko, D.; Prylutska, S.; Vareniuk, I.; Bogutska, K.; Braniuk, S.; Korotkyi, O.; Prylutsky, Y.; Ritter, U.; Piosik, J. The effect of C₆₀ fullerene on the mechanokinetics of *muscle gastrocnemius* contraction in chronically alcoholized rats. *Heliyon* **2023**, *9*, e18745. [\[CrossRef\]](#) [\[PubMed\]](#)
- Prylutska, S.V.; Grebinyk, A.G.; Lynchak, O.V.; Byelinska, I.V.; Cherepanov, V.V.; Tauscher, E.; Matyshevska, O.; Prylutsky, Y.; Rybalchenko, V.; Ritter, U.; et al. In vitro and in vivo toxicity of pristine C₆₀ fullerene aqueous colloid solution. *Fuller. Nanotub. Carbon Nanostruct* **2019**, *27*, 715–728. [\[CrossRef\]](#)
- Song, K.; Coleman, R.A.; Zhu, X.; Alber, C.; Ballas, Z.K.; Waldschmidt, T.J.; Cook, R.T. Chronic ethanol consumption by mice results in activated splenic T cells. *J. Leukoc. Biol.* **2002**, *72*, 1109–1116. [\[CrossRef\]](#) [\[PubMed\]](#)
- Deshpande, N.; Kandi, S.; Kumar, P.V.B.; Ramana, K.V.; Muddeshwar, M. Effect of Alcohol Consumption on Oxidative Stress Markers and its Role in the Pathogenesis and Progression of Liver Cirrhosis. *Am. J. Med. Biol. Res.* **2013**, *1*, 99–102. [\[CrossRef\]](#)
- Nyblom, H.; Berggren, U.; Balldin, J.; Olsson, R. High AST/ALT ratio may indicate advanced alcoholic liver disease rather than heavy drinking. *Alcohol. Alcohol.* **2004**, *39*, 336–339. [\[CrossRef\]](#)
- Cho, E.J.; Jeong, S.-M.; Chung, G.E.; Yoo, J.-J.; Cho, Y.; Lee, K.-N.; Shin, D.W.; Kim, Y.J.; Yoon, J.-H.; Han, K.; et al. Gamma-glutamyl transferase and risk of all-cause and disease-specific mortality: A nationwide cohort study. *Sci. Rep.* **2023**, *13*, 1751. [\[CrossRef\]](#)
- Niemelä, O.; Alatalo, P. Biomarkers of alcohol consumption and related liver disease. *Scand. J. Clin. Lab. Investig.* **2010**, *70*, 305–312. [\[CrossRef\]](#)
- Golka, K.; Wiese, A. Carbohydrate-deficient transferrin (CDT)-a biomarker for long-term alcohol consumption. *J. Toxicol. Environ. Health Crit. Rev.* **2004**, *7*, 319–337. [\[CrossRef\]](#) [\[PubMed\]](#)
- Sen, C.K. Antioxidant and redox regulation of cellular signaling: Introduction. *Med. Sci. Sports Exerc.* **2001**, *33*, 368–370. [\[CrossRef\]](#)
- Ji, L.L. Modulation of skeletal muscle antioxidant defense by exercise: Role of redox signaling. *Free Radic. Biol. Med.* **2008**, *44*, 142–152. [\[CrossRef\]](#) [\[PubMed\]](#)
- Gonchar, O.O.; Maznychenko, A.V.; Bulgakova, N.V.; Vereschaka, I.V.; Tomiak, T.; Ritter, U.; Prylutsky, Y.; Mankovska, I.M.; Kostyukov, A.I. C₆₀ Fullerene Prevents Restraint Stress-Induced Oxidative Disorders in Rat Tissues: Possible Involvement of the Nrf2/ARE-Antioxidant Pathway. *Oxid. Med. Cell Longev.* **2018**, *2018*, 2518676. [\[CrossRef\]](#) [\[PubMed\]](#)
- Schukit, M.A. Ethanol and methanol. In *Goodman & Gillman's Pharmacological Basis of Therapeutics*, 12th ed.; Brunton, L.L., Ed.; McGraw Hill: New Delhi, India, 2011; pp. 629–647.

28. Kuznietsova, H.M.; Dziubenko, N.V.; Lynchak, O.V.; Herheliuk, T.S.; Zavalny, D.K.; Remeniak, O.V.; Prylutsky, Y.; Ritter, U. Effects of Pristine C₆₀ Fullerenes on Liver and Pancreas in α -Naphthylisothiocyanate-Induced Cholangitis. *Dig. Diss. Sci.* **2020**, *65*, 215–224. [[CrossRef](#)] [[PubMed](#)]
29. Preedy, V.R.; Patel, V.B.; Reilly, M.E.; Richardson, P.J.; Falkous, G.; Mantle, D. Oxidants, antioxidants and alcohol: Implications for skeletal and cardiac muscle. *Front. Biosci.* **1999**, *4*, e58–e66. [[CrossRef](#)] [[PubMed](#)]
30. Preedy, V.R.; Adachi, J.; Asano, M.; Koll, M.; Mantle, D.; Niemela, O.; Parkkila, S.; Paice, A.G.; Peters, T.; Rajendram, R.; et al. Free radicals in alcoholic myopathy: Indices of damage and preventive studies. *Free Radic. Biol. Med.* **2002**, *32*, 683–687. [[CrossRef](#)]
31. Galicia-Moreno, M.; Gutiérrez-Reyes, G. The role of oxidative stress in the development of alcoholic liver disease. *Rev. Gastroenterol. Mex.* **2014**, *79*, 135–144. [[CrossRef](#)]
32. Otis, J.S.; Guidot, D.M. Procyteine increases alcohol-depleted glutathione stores in rat plantaris following a period of abstinence. *Alcohol. Alcohol.* **2010**, *45*, 495–500. [[CrossRef](#)] [[PubMed](#)]
33. Ritter, U.; Prylutsky, Y.I.; Evstigneev, M.P.; Davidenko, N.A.; Cherepanov, V.V.; Senenko, A.I.; Marchenko, O.A.; Naumovets, A.G. Structural features of highly stable reproducible C₆₀ fullerene aqueous colloid solution probed by various techniques. *Fuller. Nanotub. Carbon Nanostruct* **2015**, *23*, 530–534. [[CrossRef](#)]
34. Collins, M.A.; Neafsey, E.J. Alcohol, Excitotoxicity and Adult Brain Damage: An Experimentally Unproven Chain-of-Events. *Front. Mol. Neurosci.* **2016**, *9*, 8. [[CrossRef](#)] [[PubMed](#)]
35. D'Souza El-Guindy, N.B.; Kovacs, E.J.; De Witte, P.; Spies, C.; Littleton, J.M.; De Villiers, W.J.S.; Lott, A.J.; Plackett, T.P.; Lanzke, N.; Meadows, G.G. Laboratory models available to study alcohol-induced organ damage and immune variations: Choosing the appropriate model. *Alcohol. Clin. Exp. Res.* **2010**, *34*, 1489–1511. [[CrossRef](#)] [[PubMed](#)]
36. Gharbi, N.; Pressac, M.; Hadchouel, M.; Szwarc, H.; Wilson, S.R.; Moussa, F. [60]fullerene is a powerful antioxidant in vivo with no acute or subacute toxicity. *Nano Lett.* **2005**, *5*, 2578–2585. [[CrossRef](#)] [[PubMed](#)]
37. Nikolic, N.; Vranjes-Ethuric, S.; Jankovic, D.; Ethokic, D.; Mirkovic, M.; Bibic, N.; Trajkovic, V. Preparation and biodistribution of radiolabeled fullerene C₆₀ nanocrystals. *Nanotechnology* **2009**, *20*, 385102. [[CrossRef](#)] [[PubMed](#)]
38. Motuziuk, O.; Nozdrenko, D.; Prylutska, S.; Bogutska, K.; Korotkyi, O.; Prylutsky, Y. Biochemical parameters of blood and tissue of the muscle gastrocnemius in chronically alcoholized rats under oral administration of C₆₀ fullerene aqueous solution. *Ukr. Biochem. J.* **2023**, *95*, 58–67. [[CrossRef](#)]
39. Fentener van Vlissingen, J.M.; Borrens, M.; Girod, A.; Lelovas, P.; Morrison, F.; Torres, Y.S. The reporting of clinical signs in laboratory animals. FELASA Working Group Report. *Lab. Anim.* **2015**, *49*, 267–283. [[CrossRef](#)]
40. Suvarna, S.K.; Layton, C.; Bancroft, J.D. *Bancroft's Theory and Practice of Histological Techniques*, 8th ed.; Elsevier Limited: Amsterdam, The Netherlands, 2019; 519p.
41. Zhang, S.; Wang, C. Precise Analysis of Nanoparticle Size Distribution in TEM Image. *Methods Protoc.* **2023**, *6*, 63. [[CrossRef](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Effect of C₆₀ Fullerene on Muscle Injury-Induced Rhabdomyolysis and Associated Acute Renal Failure

Yuriy Prylutsky¹, Dmytro Nozdrenko¹, Olexandr Omelchuk², Svitlana Prylutska³, Olexandr Motuziuk^{1,2}, Vasil Soroca¹, Igor Vareniuk¹, Viktoria Stetska¹, Kateryna Bogutska¹, Uwe Ritter⁴, Jacek Piosik⁵

¹ESC "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv, Kyiv, Ukraine; ²Faculty of Biology and Forestry, Lesya Ukrainka Volyn National University, Lutsk, Ukraine; ³Faculty of Plant Protection, Biotechnology and Ecology, National University of Life and Environmental Science of Ukraine, Kyiv, Ukraine; ⁴Institute of Chemistry and Biotechnology, Technical University of Ilmenau, Ilmenau, Germany; ⁵Intercollegiate Faculty of Biotechnology, University of Gdansk, Gdańsk, Poland

Correspondence: Jacek Piosik, Email jacek.piosik@ug.edu.pl

Introduction: Rhabdomyolysis, as an acute stage of myopathy, causes kidney damage. It is known that this pathology is caused by the accumulation of muscle breakdown products and is associated with oxidative stress. Therefore, the present study evaluated the effect of intraperitoneal administration (dose 1 mg/kg) of water-soluble C₆₀ fullerenes, as powerful antioxidants, on the development of rat kidney damage due to rhabdomyolysis caused by mechanical trauma of the *muscle soleus* of different severity (crush syndrome lasting 1 min under a pressure of 2.5, 3.5, and 4.5 kg/cm², respectively).

Methods: Using tensometry, biochemical and histopathological analyses, the biomechanical parameters of *muscle soleus* contraction (contraction force and integrated muscle power), biochemical indicators of rat blood (concentrations of creatinine, creatine phosphokinase, urea and hydrogen peroxide, catalase and superoxide dismutase activity), glomerular filtration rate and fractional sodium excretion value, as well as pathohistological and morphometric features of muscle and kidney damages in rats on days 1, 3, 6 and 9 after the initiation of the injury were studied.

Results: Positive changes in biomechanical and biochemical parameters were found during the experiment by about 27–30 ± 2%, as well as a decrease in pathohistological and morphometric features of muscle and kidney damages in rats treated with water-soluble C₆₀ fullerenes.

Conclusion: These findings indicate the potential application of water-soluble C₆₀ fullerenes in the treatment of pathological conditions of the muscular system caused by rhabdomyolysis and the associated oxidative stress.

Keywords: *muscle soleus*, C₆₀ fullerene, rhabdomyolysis, biomechanical parameters of muscle contraction, blood biochemical indicators, histopathological analysis

Introduction

Acute renal failure (ARF) is a condition in which there is a rapid decline in kidney function, in particular, there is a decrease or even complete absence of urine production, which leads to the accumulation of nitrogen metabolism products in the blood. Recently, a significant number of ARF cases have been associated with rhabdomyolysis of skeletal muscle. Rhabdomyolysis is a clinical syndrome of muscle disorder caused by the destruction of skeletal muscle fibers, large artery occlusion, epileptic status, infections, etc. It is characterized by the release of skeletal muscle cell contents into the bloodstream, including myoglobin, labile iron ions, and various toxic metabolites,¹ followed by a potentially lethal accumulation of toxins, including potassium, lactic acid, and myoglobin, leading to multiple organ failure, primarily acute kidney injury.² This pathology affects up to 46% of hospitalized patients and 80% of patients requiring intensive care for rhabdomyolysis.³ Even with effective medical care, the mortality rate exceeds 25%.⁴ Over the past

decade, the incidence of rhabdomyolysis has increased 10-fold,⁵ particularly for wounded military personnel due to ballistic trauma to skeletal muscle,^{6,7} who are 5 times more likely to develop rhabdomyolysis than civilians.⁸ Global mortality from rhabdomyolysis is estimated at 10% to 46% depending on the severity, cause, early treatment, and the presence of comorbidities and complications.⁹

Currently, there is no specific treatment for rhabdomyolysis. Dialysis remains the main treatment for patients with ARF caused by rhabdomyolysis. Dialysis is believed to remove myoglobin released from damaged muscles and metabolic toxins from the bloodstream. Standard medical interventions are usually massive hydration, urine alkalization, and forced diuresis. It is reported that 28% to 65% of patients who develop rhabdomyolysis require mandatory dialysis therapy.¹⁰

Note that traumas of various natures can cause acute kidney injury (AKI). It is the early diagnosis of AKI, in particular, thanks to the use of nanoparticles¹¹ and recently discovered biomarkers,¹² ensures its effective therapy in the future. The burst of reactive oxygen species (ROS) plays a key role in the pathological progression of AKI. Therefore, mitochondrial-targeted antioxidant therapy is very promising because mitochondria are the main source of ROS in AKI. It was shown that antioxidant nanodrugs, in particular ultra-small Tungsten-based nanodots, with actively targeted mitochondria have achieved encouraging success in many oxidative stress-induced diseases.¹³ Many new antioxidant nanodrugs with intrinsic kidney targeting through the control of size, shape, and surface properties have opened exciting prospects for the treatment of AKI.^{14,15}

ARF causes a general or regional decrease in renal blood flow. Subsequent renal hypoxia and ischemia contribute to the formation of ROS, which damage biomolecules and membranes, including renal tubular cells. It has been shown that various exogenous antioxidants have a significant effect on the course of ARF induced by rhabdomyolysis. For example, ascorbate (vitamin C) reduces rhabdomyolysis kidney damage in humans.¹⁶ It has been shown that the increase in the level of lipid peroxidation products (LPO) and the activity of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase) was reversed by the administration of D-panthenol.¹⁷ Its use improved renal morphology and reduced ARF markers through antioxidant effects and normalization of mitochondrial metabolism. It was found that allopurinol administration attenuated renal dysfunction in the ARF model associated with rhabdomyolysis by reducing oxidative stress (systemic, renal and muscle), apoptosis, and inflammation. Allopurinol also reduced oxidative stress in injured muscles, attenuated muscle damage/inflammation, and accelerated muscle recovery.¹⁸ Acetaminophen has been shown to reduce kidney damage and improve kidney function by counteracting ROS and free radical formation in a rat model of rhabdomyolysis.¹⁹ Study²⁰ found that N-acetylcysteine improves renal function. It has been shown that biocompatible and bioavailable carbon nanoparticles C₆₀ fullerenes with actively targeted mitochondria²¹ are powerful scavengers of ROS induced by muscle damage: their application in vivo resulted in significant positive therapeutic effects after the initiation of ischemic injury,²² fatigue,²³ atrophy,²⁴ and skeletal muscle injury,²⁵ as well as pesticide poisoning.²⁶ Finally, in our previous work,²⁷ we demonstrated a protective effect of water-soluble C₆₀ fullerene on the development of ARF in a rat model of glycerol-induced rhabdomyolysis.

Based on the above-mentioned data, the aim of this work was to study the effect of water-soluble C₆₀ fullerenes on the development of rhabdomyolysis caused by *muscle soleus* mechanical trauma of different degrees of severity and associated ARF by evaluating biomechanical parameters of muscle contraction (contraction force and integrated muscle power) and biochemical parameters of laboratory animals blood (concentrations of creatinine, creatine phosphokinase (CPK), urea and hydrogen peroxide, CAT and SOD activity), as well as glomerular filtration rate (GFR) and fractional excretion of sodium (FE_{Na}). Additionally, the pathohistological and morphometric features of muscle and kidney damage in rats after the initiation of the injury were evaluated.

Materials and Methods

Material Preparation

An aqueous solution of pristine C₆₀ fullerene with a purity of more than 99.95% has been prepared according to the method.²⁸ Briefly, it is based on the transfer of C₆₀ molecules from toluene to water followed by sonication. Microscopic and spectroscopic data indicate that an aqueous solution of C₆₀ fullerene at a maximum concentration of 0.15 mg/mL is

a typical colloid containing both single C₆₀ molecules (~0.7 nm in diameter) as well as their nanoaggregates (up to ~100 nm)²⁹ and remains highly stable (zeta potential value is -25.3 mV³⁰) for 18 months at a temperature +4 °C.

It is important to note that due to its nanosized almost spherical shape, high chemical stability, and unique physical properties, C₆₀ fullerene is of the greatest interest for biomedical research.³¹ In particular, C₆₀ fullerene exhibits a strong reducing ability, readily attaching up to six electrons simultaneously. Due to this, it acts in *in vitro* and *in vivo* systems as a powerful scavenger of free radicals,^{32,33} the overproduction of which leads to many pathologies.

Animal Model

All experiments were performed on rats in accordance with the international principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), Article 26 of the Law of Ukraine “On the Protection of Animals from Cruelty” (No. 3447-IV, February 21, 2006) as well as the European Union Directive (2010/63/EU) “On the protection of animals used for scientific purposes”. The research protocols were approved by the Bioethics Committee of the ESC ‘Institute of Biology and Medicine’ of the Taras Shevchenko National University of Kyiv (Protocol No. 2 of September 2, 2022).

Experiments were performed on male Wistar rats weighing 150–180 g, randomly divided into the following groups:

- control group (n = 7);
- model pathology groups of 1 (n=7), 2 (n=7), and 3 (n=7) severity degrees;
- model pathology groups of 1 (n=7), 2 (n=7), and 3 (n=7) severity degrees after administration of water-soluble C₆₀ fullerenes.

Muscle trauma was induced by compressing the muscle for 1 min with a clamp under pressure of 2.5 (1st degree of severity), 3.5 (2nd degree of severity) and 4.5 (3rd degree of severity) kg/cm².³⁴ The applied crush syndrome led to a systemic manifestation of pathological changes due to the destruction of muscle cells, in particular, the release of muscle cell components (creatine kinase, myoglobin) into the extracellular environment, which served as a marker of a muscle injury severity.

It should be noted that the applied dose of water-soluble C₆₀ fullerenes of 1 mg/kg was chosen as the most effective when studying their antioxidant effect in other models of muscle pathologies.^{22–26} In addition, it is significantly lower than the LD₅₀ value, which was 600 mg/kg in the case of oral administration to rats³² and 721 mg/kg in the case of intraperitoneal administration to mice.³⁰ An aqueous solution of C₆₀ fullerene was administered intraperitoneally daily throughout the experiment, starting on the first day immediately after the initiation of muscle injury.

Animals were anesthetized by intraperitoneal injection of nembotal (40 mg/kg). The rat *soleus muscle* was freed from the surrounding tissues. Its tendon part was cut across the distal part of the muscle, which was connected to the force sensors. Efferents were stimulated with electrical pulses of 2 ms duration, generated by an ADC (analog-to-digital converter) pulse generator through platinum electrodes.^{22–26}

Biomechanical and Biochemical Analysis

In the process of analyzing the biomechanical results, the following parameters were used: minimum and maximum muscle contraction forces when performing 6 s of stimulation pool and integrated muscle power (calculated area under the force curve), which is an indicator of its overall performance.^{22–26}

The content of creatinine, CPK, urea, and hydrogen peroxide, CAT, and SOD activity in the blood of experimental animals as muscle and kidney dysfunction markers were determined using clinical diagnostic equipment - biochemical analyzers RNL-200 and JN-1101-TR2 (Netherlands).^{22–26}

GFR was determined by the clearance of endogenous creatinine:³⁵ $GFR (\mu L/min) = (V \cdot Cr_U) / Cr_B$, where V is urine volume ($\mu L/min$; minute diuresis), Cr_U ($\mu mole/l$) and Cr_B ($\mu mole/l$) are the concentrations of creatinine in urine and blood, respectively.

The FE_{Na} value (the ratio between glomerular filtration of sodium and its reabsorption) was used to assess sodium transport in the kidneys:³⁶ $FE_{Na}(\%) = 100\% \times (Cr_B \times Na_U) / (Na_B \times Cr_U)$, where Na_U and Na_B are the concentrations of sodium in urine and blood, respectively.

Histopathological Analysis

The samples of a kidney and a muscle were separated and fixed in 10% formalin, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin (H&E) for general histopathological analyses.³⁷ Sections of the muscle were additionally stained with hematoxylin and picrofuchsin by van Gieson for detecting connective tissue and muscle fibers. Digital microphotographs of stained sections were taken at $\times 100$ and $\times 400$ magnification using a computer-assisted image analyzing system (consisting of Olympus BX41 microscope and Olympus C-5050 Zoom digital camera). The histopathological profiles of the muscles and kidneys were determined by light microscopy observation. Then, the muscle fiber diameters and the area occupied by connective tissue in the muscle bundles were measured using ImageJ software. In addition, the cross-sectional area of renal glomeruli and diameter of renal tubes were measured in the kidneys using ImageJ software.

Statistical Analysis

Statistical evaluation of the results was performed using the procedure of analysis of variances (ANOVA) with mixed design. Two between-group factors were supposed: 1) injury (three levels – 1st, 2nd, and 3rd degree of severity); 2) C₆₀ fullerene treatment (two levels – no and use of C₆₀). The factor of time was supposed a within-group with four levels (1, 3, 6, and 9 days after muscle injury initiation). The Shapiro–Wilk W-test was used to test for normality. Levene's test was used to assess the equality of variances across groups. Multiple pairwise comparisons between different groups and conditions were performed by the Bonferroni *post-hoc* test. The differences between the groups were considered significant at $P < 0.05$. The statistical evaluation was performed by the software package Statistica 8.0 (Dell, USA).

Results and Discussion

Biomechanics of Muscle Contraction

Figure 1 shows the generation curves of *muscle soleus* contraction force on days 1, 3, 6, and 9 after muscle injury initiation of 1, 2, and 3 degrees of severity. Thus, on the first day after the injury, integrated muscle power decreased to $69 \pm 3\%$, $43 \pm 2\%$, and $24 \pm 1\%$ of control values at 1, 2, and 3 degrees of severity, respectively (Figure 1A and C). On the third day, these indices amounted to $29 \pm 1\%$, $17 \pm 1\%$, and $11 \pm 1\%$, respectively, in comparison with the control (Figure 1A and C), which confirms the data on the maximum dysfunction of the injured muscle on the third day after the injury.^{38,39} On the sixth day, a significant increase in the integrated power of the muscle was recorded up to $62 \pm 2\%$, $55 \pm 3\%$, and $41 \pm 3\%$ at 1, 2, and 3 degrees of severity, respectively (Figure 1A and C). Finally, on the ninth day, the value of this parameter was $83 \pm 5\%$, $79 \pm 3\%$, and $75 \pm 3\%$, respectively, relative to the control (Figure 1A and C), which is consistent with the data on the recovery of muscle function on the ninth day after injury. It should be emphasized that during a long period (2–3 months) the performance of the injured muscle does not fully recover;^{40,41} in our case, its level on the ninth day after injury initiation was $75\text{--}80 \pm 3\%$ of the control and remained so during 1 month.

The injections of C₆₀ fullerene aqueous solution increased integrated muscle power by $18 \pm 1\%$, $13 \pm 1\%$, and $14 \pm 2\%$ at severity grades 1, 2, and 3, respectively, on the first day after injury and by $23 \pm 2\%$, $18 \pm 2\%$ and $11 \pm 1\%$, respectively, on the third day (Figure 1B and C). On the sixth and ninth day, these parameters did not change significantly (Figure 1B and C) relative to the injury group. It should be noted that there was a slight difference in the positive effect of C₆₀ fullerene between the different severity levels of muscle injury on both the first and ninth days of the experiment. Thus, C₆₀ fullerene aqueous solution injections increased the level of integrated muscle power by $15\text{--}25 \pm 2\%$ relative to the injury group on the ninth day of the experiment.

When performing single-joint movements, such biomechanical indices as the generation levels of maximum and minimum force of muscle contraction are the main markers of muscle dysfunction in the development of pathological processes.^{22–26} The positive effect of C₆₀ fullerene injections on the level of minimal force (the most sensitive marker of muscle dysfunction) did not exceed $10 \pm 2\%$ relative to the injury group on the first day of the experiment (Figure 2A). On the third day (the day of most maximal dysfunction of the injured muscle), this index increased significantly and was $29 \pm 2\%$, $73 \pm 3\%$, and $75 \pm 4\%$ at severity grades 1, 2, and 3, respectively (Figure 2B). On the sixth and ninth day, it amounted to $45\text{--}50 \pm 2\%$ relative to the injury group (Figure 2C and D). The change in the level of maximal force of muscle contraction at injections of C₆₀ fullerene amounted to 12–

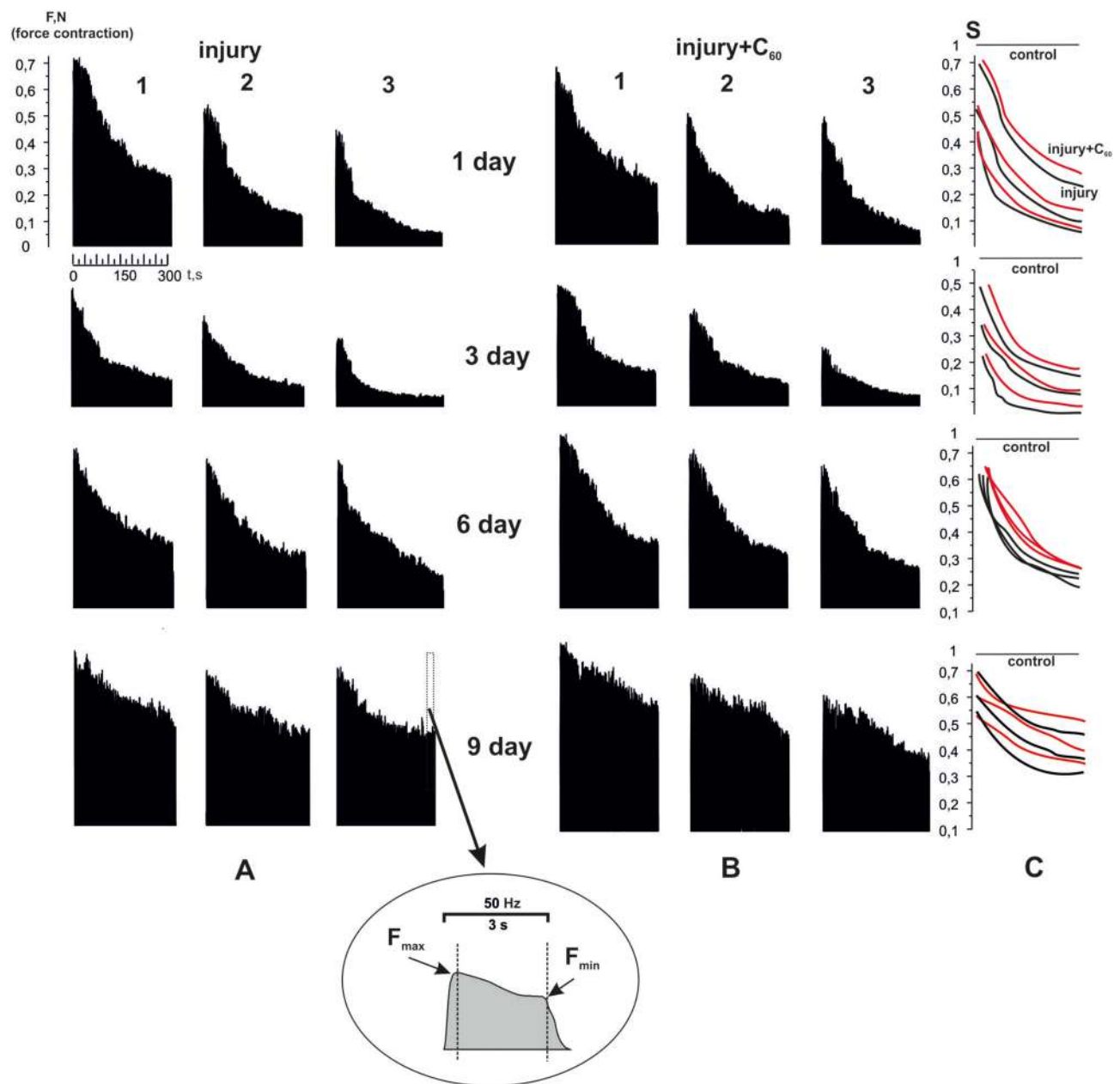


Figure 1 Muscle soleus contraction force after the injury initiation of 1, 2 and 3 degrees of severity on the 1st, 3rd, 6th and 9th day of the experiment without C₆₀ fullerene aqueous solution administration (A) and on the background of its injection (B) at stimulation by a signal of 50 Hz frequency with duration of 3 s for 300 s; F_{max} and F_{min} - maximum and minimum forces of single muscle contraction; S - integrated muscle power (in relative units) (C).

16 ± 1% relative to the injury group at all time periods of the experiment (Figure 2). The obtained data testify to the suppressive effect of the aqueous solution of C₆₀ fullerene on free-radical processes in the injured muscle, thus promoting myocytes to generate the minimum force at a higher level. The insignificant positive effect of C₆₀ fullerene on the level of maximal force generation in the injured muscle may be related to the free-radical processes that occur inside the injured myocytes and prevent actomyosin interactions.

Thus, biomechanical studies show that C₆₀ fullerene aqueous solution injections significantly reduce the level of development of pathologic processes in injured muscle, which, in turn, may influence the level of ARF-initiated rhabdomyolysis.

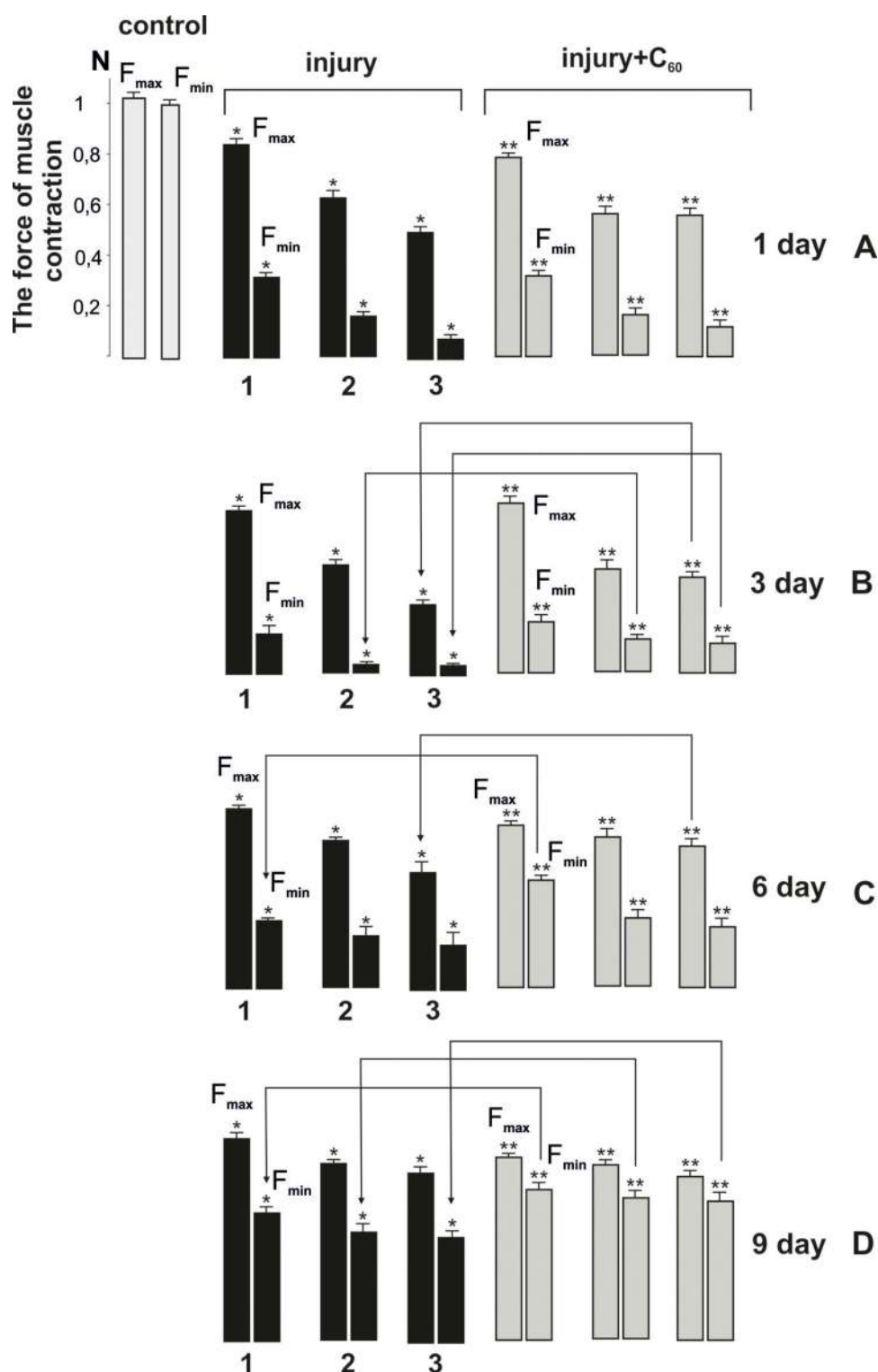


Figure 2 The biomechanical parameters of *muscle soleus* contraction after the injury initiation of 1, 2, and 3 degrees of severity on the 1st (A), 3rd (B), 6th (C) and 9th (D) day of the experiment without C₆₀ fullerene aqueous solution administration (injury) and against the background of its injections (injury + C₆₀); F_{max} and F_{min} - maximum and minimum forces of single muscle contraction; * $P < 0.05$ relative to the control group; ** $P < 0.05$ relative to the injury group. Arrows indicate the most significant differences between injury and injury+C₆₀ groups.

Biochemical Analysis

Blood creatinine level is an important marker of muscle injury as well as ARF.⁴² In rhabdomyolysis' ARF, it can increase 2 (with 1 degree of injury severity), 3 (with 2 degree of injury severity), 4 times or higher (with 3 degree of injury

severity) from baseline.² The data obtained (Figure 3A) confirm the significant increase in creatinine level with increasing severity of muscle injury. However, on the third day of the experiment, the creatinine level decreased compared to the first day after injury initiation by $11 \pm 1\%$, $14 \pm 1\%$, and $23 \pm 1\%$ at 1, 2, and 3 degrees of severity, respectively, relative to the control. In our opinion, this is due to the cessation of the release of intracellular myocyte contents into the circulating blood. On the sixth and ninth day after the injury, creatinine levels increased dramatically by

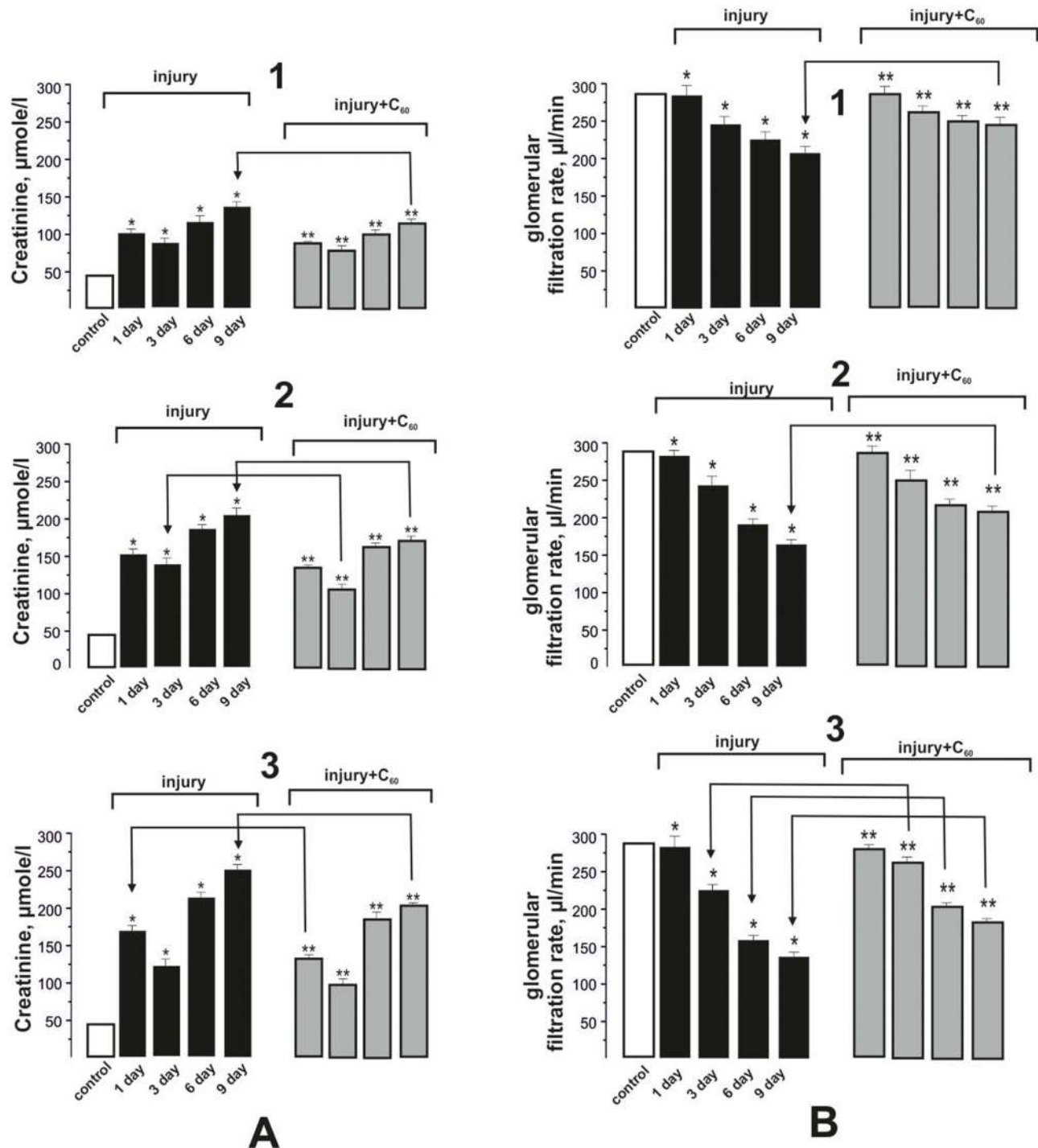


Figure 3 Blood creatinine (A) levels in rats and GFR (B) with rhabdomyolysis kidney injury: injury and injury + C₆₀ - indices after initiation of muscle injury of 1, 2, and 3 degrees of severity on the 1st, 3rd, 6th, and 9th day of the experiment without C₆₀ fullerene aqueous solution administration and on the background of its injection, respectively; **P* < 0.05 relative to the control; ***P* < 0.05 relative to the injury group. Arrows indicate the most significant differences between injury and injury+C₆₀ groups.

150–170 $\pm$ 5% and 300–450 $\pm$ 5% at 1 and 3 injury severity levels, respectively. Such an increase indicates the development of ARF caused by rhabdomyolysis.

C₆₀ fullerene aqueous solution injections lead to a 14–22 $\pm$ 1%, 21–24 $\pm$ 2%, and 30–40 $\pm$ 2% decrease in the level of creatinine at the 1st, 2nd, and 3rd degree of injury severity, respectively, relative to the injury group during the experiment (Figure 3A).

GFR is an important indicator that characterizes the ability of the kidneys to perform their main function - to clear plasma and produce urine.⁴³ During the development of rhabdomyolysis' ARF outside the proximal convoluted tubule (in the thick ascending part of the loop of Henle), urinary myoglobin combines with Tamm-Horsfall protein to form a precipitate. pH-dependent precipitate forms tubular casts that obstruct the distal tubules.⁴⁴ Obstruction is thought to increase intra-canalicular pressure above interstitial pressure, reducing vascular inflow and perfusion, promoting inflammation, and directly reducing GFR by altering Starling forces, and the effect of antioxidants on this process may reduce ARF development.^{43,44}

Decrease in GFR value was recorded starting from the third day after injury initiation (Figure 3B). On the ninth day, the difference from control values was 21 $\pm$ 1%, 43 $\pm$ 4%, and 67 $\pm$ 4% at 1, 2, and 3 degrees of injury severity, respectively. When applying C₆₀ fullerene injections, this index increased approximately by 17 $\pm$ 1%, 21 $\pm$ 1%, and 27 $\pm$ 1% at 1, 2, and 3 degrees of injury severity, respectively, relative to the injury group. At the same time, it should be noted a slight difference in the GFR values (within the error) on the sixth and ninth day of the experiment (Figure 3B).

When ARF develops, the concentration in the blood of nitrogenous slag, which can only be excreted by the body with urine, increases significantly, which is a sign of deteriorating kidney function. Urea is one of the end products of protein metabolism that contains nitrogen. The result of a blood urea test is one of the main indicators of the quality of glomerular filtration in ARF development.

An increase in urea levels was observed starting from the third day of the experiment and amounted to 83–156 $\pm$ 5%, 89–211 $\pm$ 5%, and 203–340 $\pm$ 7% of the control at 1, 2, and 3 muscle injury severity degrees, respectively, on days 3–9 of the experiment (Figure 4A). C₆₀ fullerene injections decreased urea concentration by 17–18 $\pm$ 1%, 20–22 $\pm$ 1%, and 24–27 $\pm$ 2% at 1, 2, and 3 degrees of injury severity, respectively, during the experiment (Figure 4A).

Also, one of the important quantitative indicators of the degree of ARF development is sodium transport assessment, since the mechanism of free-radical inhibition of reabsorption in rhabdomyolysis is associated with the inhibition of sodium-potassium ATPase transport system, and the action of antioxidants can reduce the development of cascade pathological reactions.⁴⁵ On the third day of the experiment, the FE_{Na} value increased by 31 $\pm$ 1%, 98 $\pm$ 3%, and 141 $\pm$ 4% relative to the control at 1, 2, and 3 injury severity, respectively (Figure 4B). On the sixth and ninth day, this index increased by 45–271 $\pm$ 5% and 128–387 $\pm$ 7% relative to the control at 2 and 3 injury severity, respectively. Injections of C₆₀ fullerene aqueous solution reduced FE_{Na} value by 13–15 $\pm$ 1%, 20–23 $\pm$ 1%, and 25–32 $\pm$ 2% at 1, 2, and 3 muscle injury severity, respectively, relative to the injury group during the experiment (Figure 4B).

Thus, the application of C₆₀ fullerene demonstrated significant (15–35 $\pm$ 2%) positive effects on the main markers of ARF induced by rhabdomyolysis. Note that their most significant reduction is observed in severe forms of rhabdomyolysis on the ninth day after muscle injury initiation.

Recently, CPK concentration measurement in blood has become a clinical test to determine the level of rhabdomyolysis development due to its rapidity and cheapness.⁵ The relationship between CPK level increase and ARF development in patients with muscle injuries has been established.⁴⁶ CPK is an enzyme from the energy supply system of skeletal muscle cells, which catalyzes the transfer of phosphate group from ATP to creatinine molecule to form the high-energy compound creatinine phosphate. In case of mechanical muscle damage, there is a release of this enzyme from the cells and, accordingly, an increase in its concentration in the blood.

CPK levels on the first day after injury rose by 112 $\pm$ 3%, 234 $\pm$ 7%, and 414 $\pm$ 7% compared with control values at 1, 2, and 3 degrees of muscle injury severity, respectively (Figure 5A). CPK levels decreased on the following days of the experiment and on the ninth day were 13–25 $\pm$ 1% of control values, despite an increase in ARF development on the sixth and ninth days.

C₆₀ fullerene injections significantly reduced CPK levels, which were 24 $\pm$ 1%, 31 $\pm$ 2% and 34 $\pm$ 2% and 17 $\pm$ 1%, 36 $\pm$ 2% and 42 $\pm$ 2% at 1, 2, and 3 degrees of muscle injury severity, respectively, on the first and third day of the

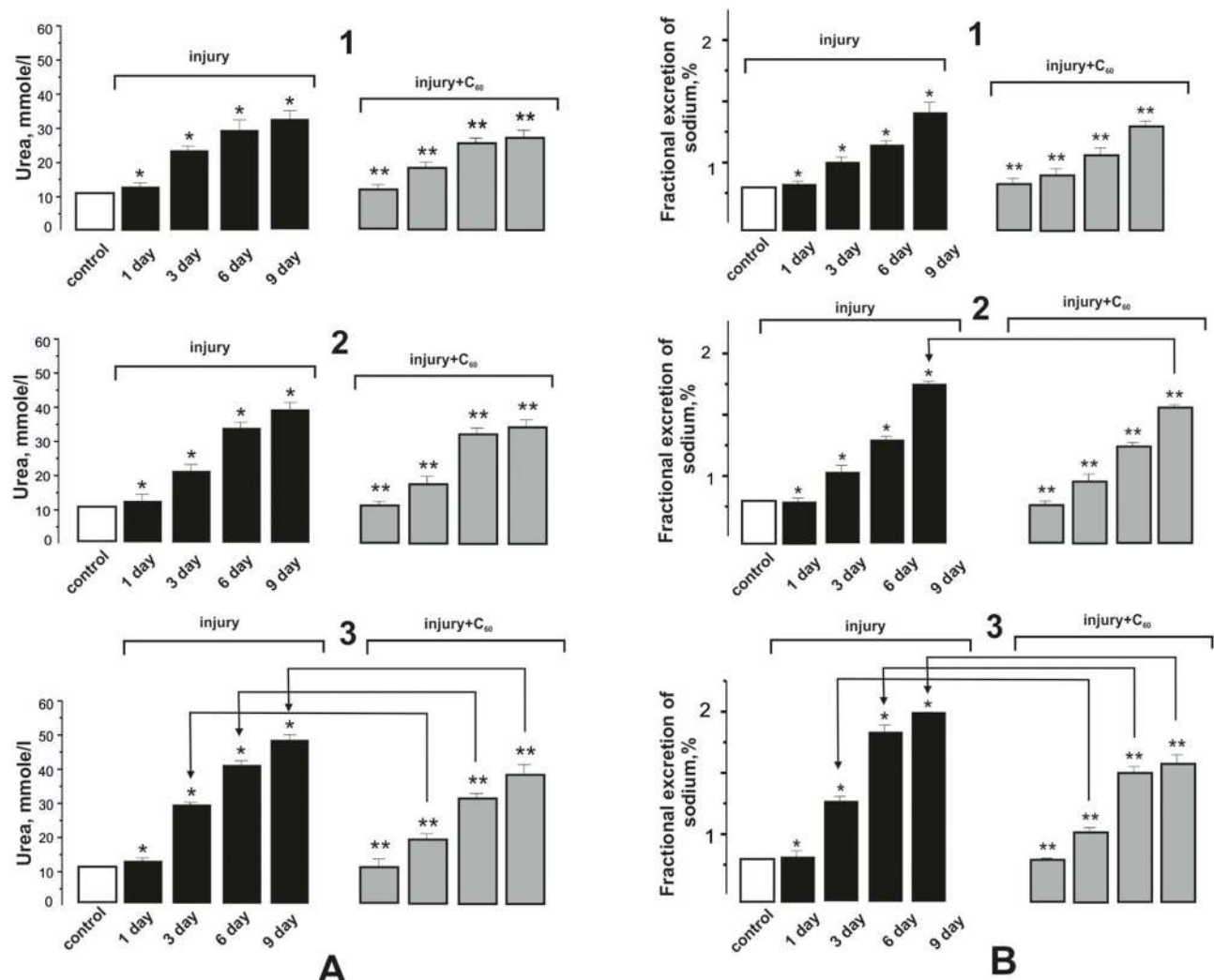


Figure 4 Blood urea (A) levels in rats and FE_{Na} (B) in rhabdomyolysis kidney injury: injury and injury+C₆₀ - indices after muscle injury initiation of 1, 2, and 3 degrees of severity on the 1st, 3rd, 6th, and 9th day of the experiment without C₆₀ fullerene aqueous solution administration and on the background of its injection, respectively; * $P < 0.05$ relative to the control; ** $P < 0.05$ relative to the injury group. Arrows indicate the most significant differences between injury and injury+C₆₀ groups.

experiment relative to the injury group. On the ninth day, the positive effect was insignificant due to the small level of CPK increase relative to the control during this period (Figure 5A).

Pathological inflammatory processes occurring immediately after injury are a source of ROS and contribute to the intensification of LPO processes.^{38,47} During the physiological recovery of muscle tissue, oxygen entering the tissues initiates xanthine and hypoxanthine oxidation by xanthine oxidase, which leads to the formation of a large amount of superoxide anion radical and hydrogen peroxide. In addition, inflammatory processes increase the expression of adhesive molecules on the endothelium. Activated neutrophils attracted to the focus of damage also release hydrogen peroxide and provoke pathological free-radical processes.

Throughout the experiment, an increase in the level of H₂O₂ was recorded, which is associated with the muscle fiber destruction and the development of inflammatory processes. The increase in H₂O₂ concentration on the first and third day was $24-28 \pm 1\%$, $42-79 \pm 2\%$, and $98-124 \pm 3\%$ at 1, 2, and 3 injury severity levels, respectively, relative to the control (Figure 5B). Further increases in H₂O₂ concentration of $10 \pm 1\%$, $14 \pm 1\%$ and $19 \pm 1\%$ and $15 \pm 1\%$, $26 \pm 2\%$ and $33 \pm 2\%$ on the sixth and ninth day of the experiment, respectively, indicated the development of rhabdomyolysis' ARF.

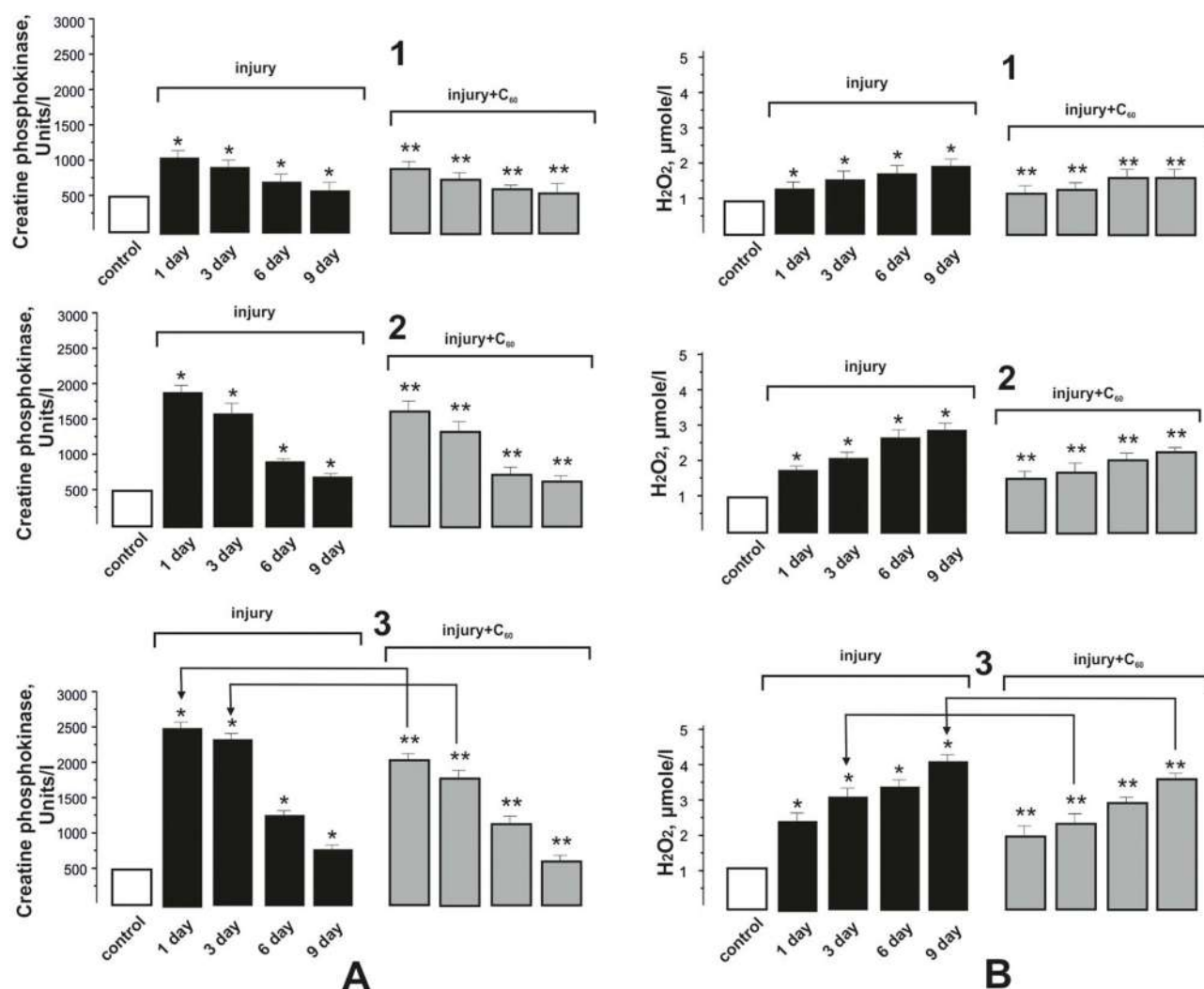


Figure 5 Levels of CPK (**A**) and H₂O₂ (**B**) in the blood of rats at rhabdomyolysis kidney injury: injury and injury + C₆₀ - indices after muscle injury initiation of 1, 2 and 3 degrees of severity on the 1st, 3rd, 6th and 9th day of the experiment without C₆₀ fullerene aqueous solution administration and on the background of its injection, respectively; **P* < 0.05 relative to the control; ***P* < 0.05 relative to the injury group. Arrows indicate the most significant differences between injury and injury+C₆₀ groups.

At C₆₀ fullerene aqueous solution injections there is a decrease in H₂O₂ concentration by 11–12 ± 1%, 15–18 ± 1%, and 22–29 ± 1% at 1, 2, and 3 degrees of muscle injury severity, respectively, relative to the injury group during the experiment (Figure 5B).

The increase in H₂O₂ level during muscle fiber injury leads to an increase in CAT activity, which performs a protective antioxidant function by catalyzing the decomposition of hydrogen peroxide into water, oxygen and SOD. The most powerful natural antioxidant and enzyme of the first link of antioxidant defense is SOD, which performs the dismutation reaction of superoxide anion radicals and transforms them into less reactive hydrogen peroxide molecules.

SOD activity on the first day of the experiment increased by 25 ± 1%, 39 ± 4%, and 45 ± 2% at the 1st, 2nd, and 3rd degree of muscle injury severity, respectively, relative to the control (Figure 6A). On the third day, this index decreased by 11–14 ± 1%, which seems to be associated with a decrease in inflammatory processes in the muscle tissue. However, on the sixth and ninth day, SOD activity increased dramatically by 23–29 ± 1%, 39–41 ± 2%, and 56–59 ± 2% at injury severity grades 1, 2, and 3, respectively, compared to the third day of the experiment, which is most likely due to the development of ARF caused by rhabdomyolysis.

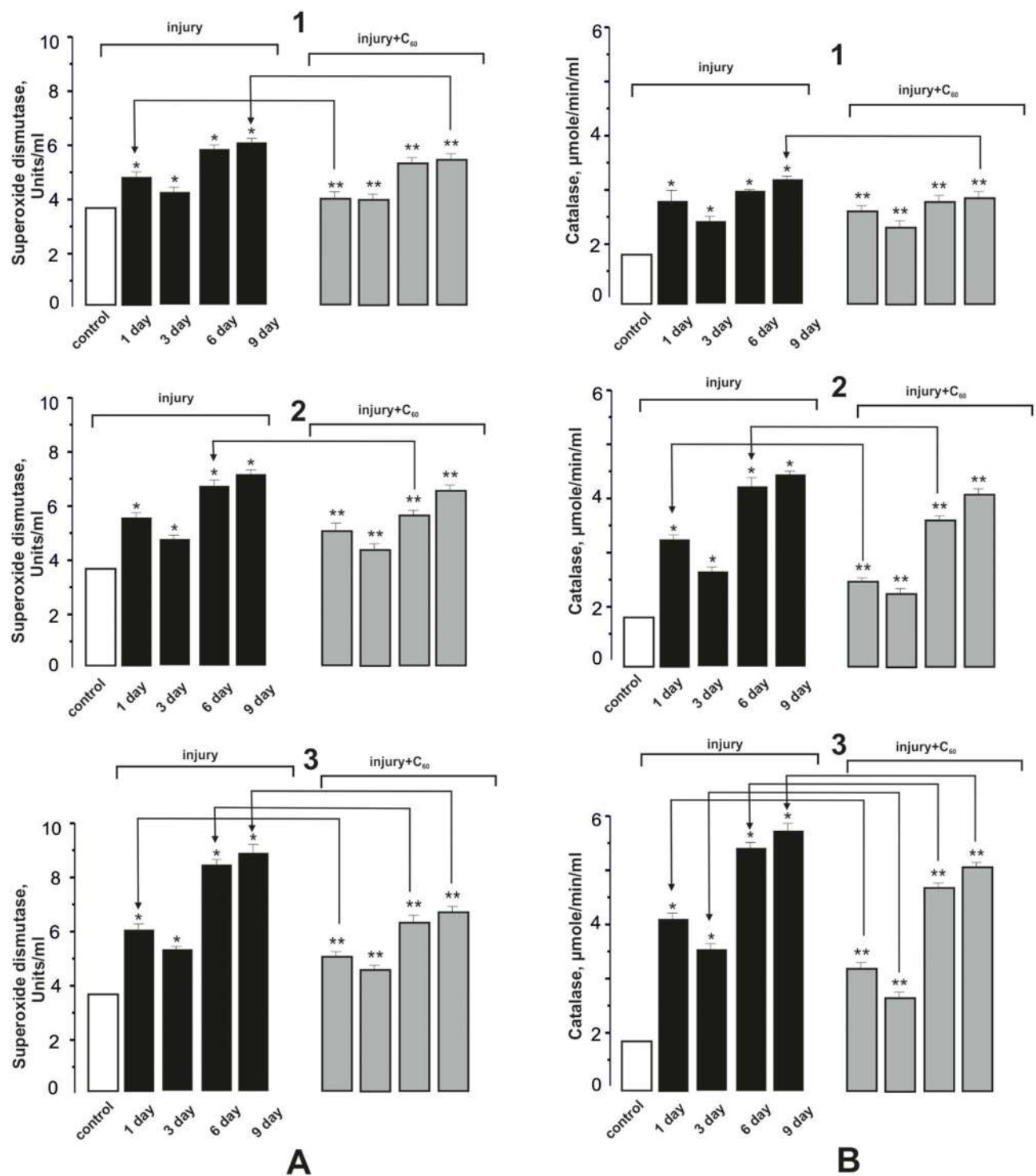


Figure 6 Activity of SOD (A) and CAT (B) in the blood of rats at rhabdomyolysis kidney injury: injury and injury + C₆₀ - indices after muscle injury initiation of 1, 2, and 3 degrees of severity on the 1st, 3rd, 6th, and 9th day of the experiment without C₆₀ fullerene aqueous solution administration and on the background of its injection, respectively; **P* < 0.05 relative to the control; ***P* < 0.05 relative to the injury group. Arrows indicate the most significant differences between injury and injury+C₆₀ groups.

C₆₀ fullerene injections decreased SOD activity by $12 \pm 1\%$ and $15 \pm 1\%$ relative to the injury group on the first and third day of the experiment. On the sixth and ninth day, the positive effects of C₆₀ fullerene injections were $10 \pm 1\%$, $15 \pm 1\%$, and $29 \pm 2\%$ at 1, 2, and 3 degrees of muscle injury severity, respectively, relative to the injury group (Figure 6A).

CAT activity on the first day of the experiment increased by $111 \pm 6\%$, $123 \pm 6\%$, and $215 \pm 8\%$ at 1, 2, and 3 degrees of muscle injury severity, respectively, relative to the control (Figure 6B). On the third day, this index, as well as SOD activity, decreased by $15 \pm 1\%$, $27 \pm 2\%$, and $34 \pm 6\%$, which seems to be associated with a decrease in inflammatory processes in muscle tissue. On the sixth and ninth day, CAT activity increased sharply by $27\text{--}31 \pm 2\%$, $65\text{--}79 \pm 3\%$, and $81\text{--}94 \pm 3\%$ at 1, 2, and 3 injury severity levels, respectively, compared to the third day of the experiment.

The positive therapeutic effect of C_{60} fullerene aqueous solution injections on CAT activity was within the changes of SOD activity in all investigated time ranges and degrees of muscle injury (Figure 6A and B). So, C_{60} fullerene injections decreased CAT activity by $11 \pm 1\%$, $24 \pm 1\%$, and $33 \pm 2\%$ relative to the injury group on day one and by $10 \pm 1\%$, $11 \pm 1\%$, and $14 \pm 1\%$ on day three of the experiment. On the sixth and ninth day, the positive effects of C_{60} fullerene injections were $10 \pm 1\%$, $12 \pm 2\%$ and $16 \pm 2\%$ and $10 \pm 1\%$, $11 \pm 1\%$ and $14 \pm 1\%$ at 1, 2, and 3 degrees of muscle injury severity, respectively, relative to the injury group (Figure 6B).

Histopathological Analysis

Muscles

No deviations from the normal histological structure of the muscle were observed in the control group. Groups of muscle fibers are visible, separated by thin layers of connective tissue. Inside each muscle fiber, myofibrils are clearly visible (Figure 7A).

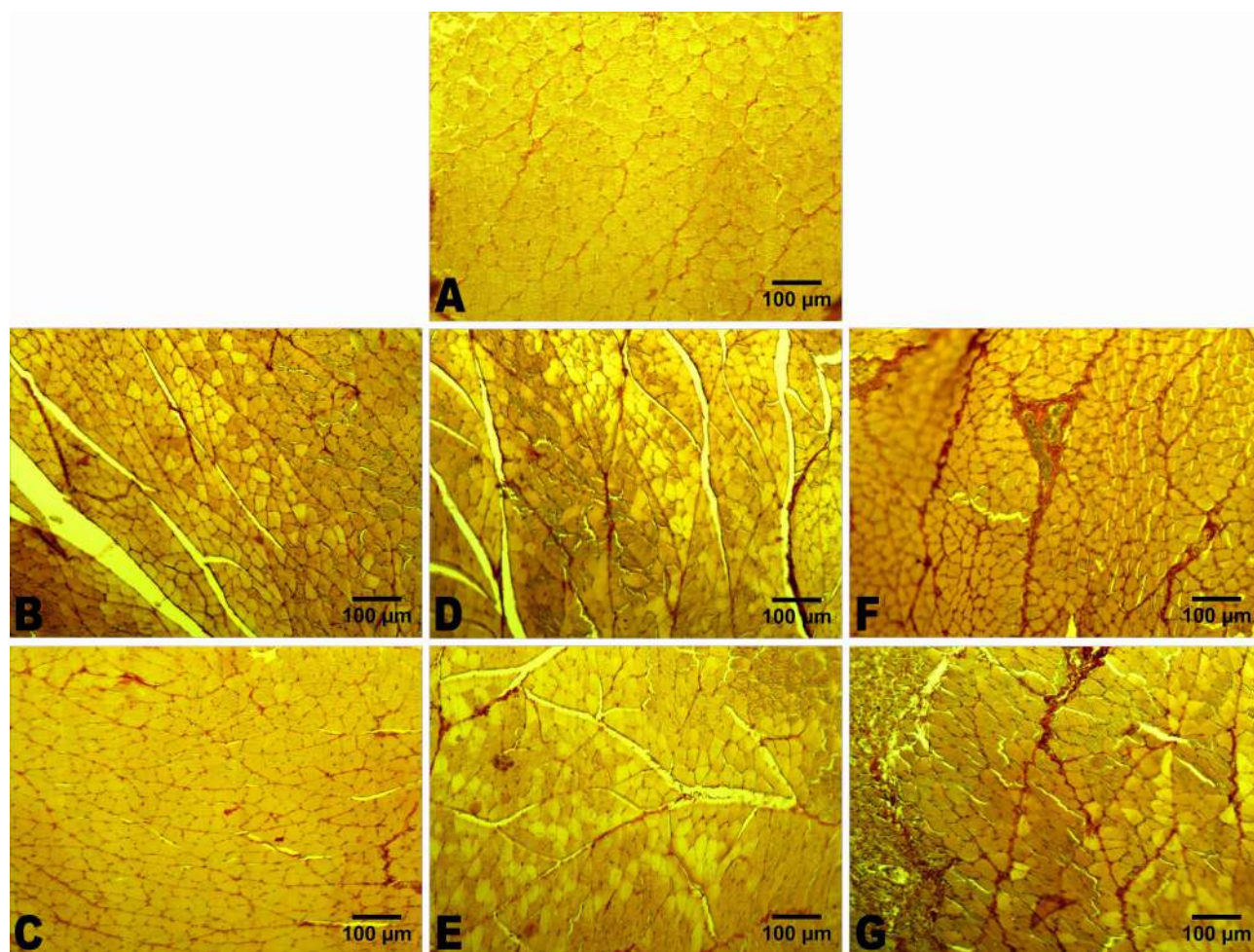


Figure 7 Representative histological images of the *muscle soleus*: (A) – control group; (B) – muscle injury (1st degree of severity); (C) – muscle injury (1st degree of severity) + C_{60} ; (D) – muscle injury (2nd degree of severity); (E) – muscle injury (2nd degree of severity) + C_{60} ; (F) – muscle injury (3rd degree of severity); (G) – muscle injury (3rd degree of severity) + C_{60} . Hematoxylin and picrofuchsin staining by van Gieson. Scale bars – 100 µm.

In rats with muscle injury, muscle fibers are destroyed and connective tissue is heavily proliferated at the site of injury (Figure 7B, D and F). In other parts of the muscle, the normal histological structure of the muscle fibers is disturbed. Some of the fibers are hypochromatic, with the signs of edema. Some fibers are hyperchromatic, with the signs of destruction. The structure of myofibrils inside the muscle fibers is disturbed. There is a disturbance in the transverse striation of the fiber. Fibers may decrease in diameter. Connective tissue also grows: the area occupied by connective tissue increases in all study groups (Table 1). In cases of the 1st and the 2nd degrees of muscle injury severity (Figure 7B and D), the increase in connective tissue volume is mainly due to the matrix ground substance of connective tissue, and in the case of the 3rd degree (Figure 7F) – mainly due to the growth of collagen fibers. The severity of the above pathological and morphometric changes varies in different experimental groups (Table 1).

The area occupied by connective tissue in rats with the 1st degree of muscle injury severity is $80\text{--}110 \pm 7\%$ higher than in the control group. The diameter of muscle fibers is $16 \pm 2\%$ smaller compared to the control group in rats that did not receive water-soluble C₆₀ fullerenes but does not differ from the control values in rats that received them (Table 1).

In rats with the 2nd degree of muscle injury severity, which received water-soluble C₆₀ fullerenes, the area occupied by connective tissue is $20 \pm 2\%$ smaller and the diameter of muscle fibers is $30 \pm 2\%$ larger (and does not differ from the control) compared to rats that did not receive them (Table 1).

In rats with the 3rd degree of muscle injury severity, which received water-soluble C₆₀ fullerenes, the area occupied by connective tissue is $31 \pm 2\%$ smaller and the diameter of muscle fibers is $12 \pm 1\%$ larger compared to rats that did not receive them (Table 1).

Thus, in rats treated with water-soluble C₆₀ fullerenes, connective tissue does not grow so much, and the diameter of muscle fibers does not decrease (in cases of the 1st and 2nd severity of muscle injury), or decreases to a lesser extent (in case of the 3rd severity of muscle injury). The degree of destructive pathological changes in muscle tissue is also less (Figure 7C, E and G).

Kidney

In control rats, the kidneys have a normal histological structure (Figure 8A). In rats with muscle injury (Figure 8B, D and F), retraction of glomerular tuft is observed in part of the renal glomeruli. As a result, the cross-sectional area of the renal glomeruli decreases. In addition, in cases of 3rd degree of muscle injury severity (Figure 8F), a slight hemorrhage is observed in some renal glomeruli. The diameter of the renal tubes may decrease (Table 1).

Thus, noticeable histopathological changes in the kidneys are not observed after muscle injury. However, morphometric parameters may decrease (this is evidence of a decrease in the functional activity of the kidney). The severity of

Table 1 Morphometrical Features of Muscle and Kidney in Tested Rats

Groups	Morphometrical Features	Muscle		Kidney	
		Diameter of Muscle Fibers, μm	Area occupied by Connective Tissue, $\mu\text{m}^2/\mu\text{m}^2$	Cross-sectional Area of Renal Glomeruli, μm^2	Diameter of Renal Tubes, μm
Control		30.2 ± 1.0	0.10 ± 0.01	4848 ± 217	36.1 ± 1.3
muscle injury (1 st degree of severity)		$25.4 \pm 1.1^*$	$0.21 \pm 0.02^*$	$2723 \pm 149^*$	$32.2 \pm 1.1^*$
muscle injury (1 st degree of severity) + C ₆₀		$29.5 \pm 1.5^\wedge$	$0.18 \pm 0.01^\wedge$	$3681 \pm 205^\wedge$	$35.0 \pm 1.1^\wedge$
muscle injury (2 nd degree of severity)		$21.6 \pm 0.4^*$	$0.25 \pm 0.03^*$	$3853 \pm 163^*$	$32.8 \pm 1.0^*$
muscle injury (2 nd degree of severity) + C ₆₀		$28.0 \pm 0.9^\wedge$	$0.20 \pm 0.01^\wedge$	$3460 \pm 116^\wedge$	$34.3 \pm 1.0^\wedge$
muscle injury (3 rd degree of severity)		$23.2 \pm 0.6^*$	$0.22 \pm 0.02^*$	$3572 \pm 217^*$	$29.0 \pm 1.1^*$
muscle injury (3 rd degree of severity) + C ₆₀		$26.0 \pm 1.4^\wedge$	$0.15 \pm 0.01^\wedge$	$3629 \pm 228^\wedge$	$31.7 \pm 1.0^\wedge$

Notes: * $P < 0.05$ compared with the control group; $^\wedge P < 0.05$ compared with the muscle injury group at the same degree of severity.

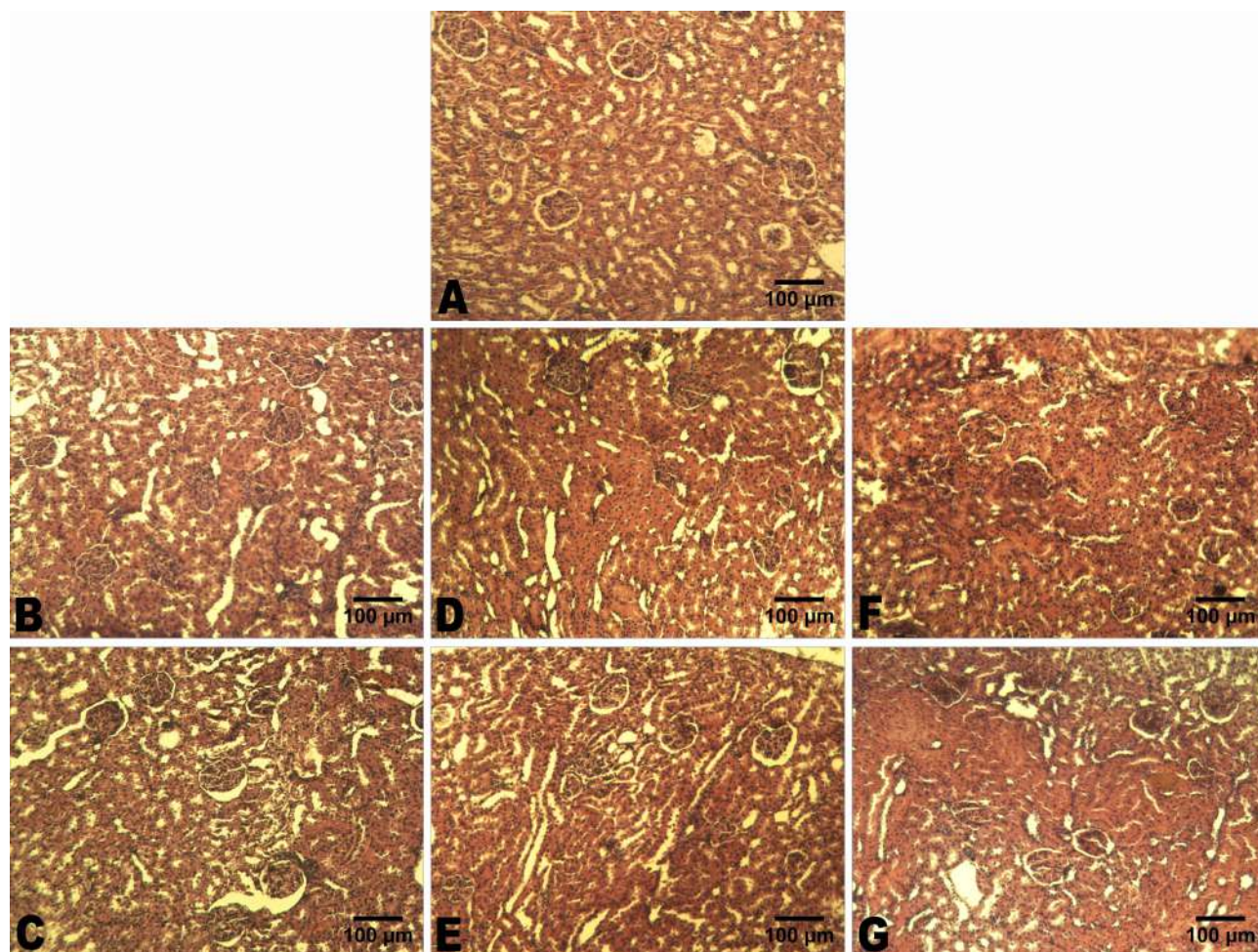


Figure 8 Representative histological images of the kidney: (A) – control group; (B) – muscle injury (1st degree of severity); (C) – muscle injury (1st degree of severity) + C₆₀; (D) – muscle injury (2nd degree of severity); (E) – muscle injury (2nd degree of severity) + C₆₀; (F) – muscle injury (3rd degree of severity); (G) – muscle injury (3rd degree of severity) + C₆₀. H&E staining. Scale bars – 100 μm.

these changes varies in the different experimental groups. For example, the diameter of renal tubules in rats with 3rd degree of muscle injury severity, which received water-soluble C₆₀ fullerenes, is bigger by $10 \pm 1\%$ compared to rats with injury (Table 1; Figure 8G), which did not receive them. In rats with 1st and 2nd degrees of muscle injury severity, which received water-soluble C₆₀ fullerenes, the diameter of renal tubules did not differ from the control values (Table 1; Figure 8C and E).

Summarizing, the above-described positive effects of exposure to C₆₀ fullerenes on the ARF caused by rhabdomyolysis are related precisely to their powerful antioxidant properties.^{25,26} C₆₀ molecules effectively inactivate the ROS, protecting nephron membranes from oxidation, and, thus, reduce the severity of ARF.

Conclusion

Thus, during the experiment, there is a positive change of the studied biomechanical and biochemical parameters by about $27\text{--}30 \pm 2\%$ with C₆₀ fullerene aqueous solution administration at a low therapeutic dosage. Besides, they were confirmed by the data of histopathological analysis. This indicates that there is compensatory activation by C₆₀ fullerenes of the endogenous antioxidant system of the organism in the process of posttraumatic changes of the *muscle soleus* caused by mechanical damage of different degrees of severity. Thus, C₆₀ fullerenes, as powerful antioxidants, reduce the development of both rhabdomyolysis and ARF induced by it. We can also assume the therapeutic effect of C₆₀ fullerenes on the acceleration of the process of repair of muscle structures damaged by rhabdomyolysis, which is confirmed by the

previously obtained data on their efficient action on the functions of the antioxidant system of the organism at various pathological processes.^{22,24,25}

Based on the obtained results, it can be suggested that the use of the aqueous solution of C₆₀ fullerene as a therapeutic agent is able to correct pathological conditions of the muscular and excretory system arising from skeletal muscle injuries.

Acknowledgments

We acknowledge support for the publication costs by the Open Access Publication Fund of the University of Gdansk, Poland. This research was supported by the National Research Foundation of Ukraine (2022.01/0004).

Disclosure

The authors declare no conflicts of interest in this work.

References

- Hudkova O, Krysiuk I, Drobot L, Latyshko H. Rhabdomyolysis attenuates activity of semicarbazide sensitive amine oxidase as the marker of nephropathy in diabetic rats. *Ukr Biochem J*. 2022;94(1):23–32. doi:10.15407/ubj94.01.023
- Candela N, Silva S, Georges B, et al. Short- and long-term renal outcomes following severe rhabdomyolysis: a French multicenter retrospective study of 387 patients. *Ann Intensive Care*. 2020;10(1):27. doi:10.1186/s13613-020-0645-1
- Melli G, Chaudhry V, Cornblath DR. Rhabdomyolysis: an evaluation of 475 hospitalized patients. *Medicine*. 2005;84(6):377–385. doi:10.1097/01.md.0000188565.48918.41
- Nielsen FE, Cordtz JJ, Rasmussen TB, Christiansen CF. The Association Between Rhabdomyolysis, Acute Kidney Injury, Renal Replacement Therapy, and Mortality. *Clin Epidemiol*. 2020;12:989–995. doi:10.2147/CLEP.S254516
- Boden BP, Isaacs DJ, Ahmed AE, Anderson SA. Epidemiology of Exertional Rhabdomyolysis in the United States: analysis of NEISS Database 2000 to 2019. *Phys Sportsmed*. 2022;50(6):486–493. doi:10.1080/00913847.2021.1956288
- Shin EH, Sabino JM, Nanos GP, Valerio IL. Ballistic trauma: lessons learned from Iraq and Afghanistan. *Semin Plast Surg*. 2015;29(01):10–19. doi:10.1055/s-0035-1544173
- Division AF. Exertional rhabdomyolysis among active component members of the U.S. Armed Forces, 2019–2023. *MSMR*. 2024;31:9–14.
- Hill OT, Wahi MM, Carter R, et al. Rhabdomyolysis in the US Active Duty Army, 2004–2006. *Med Sci Sports Exerc*. 2012;44(3):442–449. doi:10.1249/MSS.0b013e3182312745
- Zimmerman JL, Shen MC. Rhabdomyolysis. *Chest*. 2013;144(3):1058–1065. doi:10.1378/chest.12-2016
- de Meijer AR, Fikkers BG, de Keijzer MH, et al. Serum creatine kinase as predictor of clinical course in rhabdomyolysis: a 5-year intensive care survey. *Intensive Care Med*. 2003;29(7):1121–1125. doi:10.1007/s00134-003-1800-5
- Ma Y, Cai F, Li Y, et al. A review of the application of nanoparticles in the diagnosis and treatment of chronic kidney disease. *Bioact Mater*. 2020;5(3):732–743. doi:10.1016/j.bioactmat.2020.05.002
- Xiao Z, Huang Q, Yang Y, et al. Emerging early diagnostic methods for acute kidney injury. *Theranostics*. 2022;12(6):2963–2986. doi:10.7150/thno.71064
- Huang Q, Yang Y, Zhao T, et al. Passively-targeted mitochondrial tungsten-based nanodots for efficient acute kidney injury treatment. *Bioact Mater*. 2022;21:381–393. doi:10.1016/j.bioactmat.2022.08.022
- Wang L, Zhang Y, Li Y, et al. Recent advances in engineered nanomaterials for acute kidney injury theranostics. *Nano Res*. 2021;14(4):920–933. doi:10.1007/s12274-020-3067-3
- Chen Q, Nan Y, Yang Y, et al. Nanodrugs alleviate acute kidney injury: manipulate RONS at kidney. *Review Bioact Mater*. 2022;22:141–167. doi:10.1016/j.bioactmat.2022.09.021
- Dennis JM, Witting PK. Protective role for antioxidants in acute kidney disease. *Nutrients*. 2017;9(7):718. doi:10.3390/nu9070718
- Semenovich DS, Plotnikov EY, Lukiyenko EP, et al. Protective effect of D-panthenol in rhabdomyolysis-induced acute kidney injury. *Int J Mol Sci*. 2022;23(20):12273. doi:10.3390/ijms232012273
- Gois PHF, Canale D, Volpini RA, et al. Allopurinol attenuates rhabdomyolysis-associated acute kidney injury: renal and muscular protection. *Free Radic Biol Med*. 2016;101:176–189. doi:10.1016/j.freeradbiomed.2016.10.012
- Heard KJ. Acetylcysteine for Acetaminophen poisoning. *N Engl J Med*. 2008;359(3):285–292. doi:10.1056/NEJMc0708278
- Shimizu MHM, Coimbra TM, de Araujo M, et al. N-acetylcysteine attenuates the progression of chronic renal failure. *Kidney Int*. 2005;68(5):2208–2217. doi:10.1111/j.1523-1755.2005.00677.x
- Foley S, Crowley C, Smaih M, et al. Cellular localisation of a water-soluble fullerene derivative. *Biochem Biophys Res Commun*. 2002;294(1):116–119. doi:10.1016/S0006-291X(02)00445-X
- Nozdrenko D, Matvienko T, Vygovska O, et al. Protective effect of water-soluble C₆₀ fullerene nanoparticles on the ischemia-reperfusion injury of the muscle soleus in rats. *Int J Mol Sci*. 2021;22(13):6812. doi:10.3390/ijms22136812
- Prylutsky Y, Nozdrenko D, Gonchar O, et al. C₆₀ fullerene attenuates muscle force reduction in a rat during fatigue development. *Heliyon*. 2022;8(12):e12449. doi:10.1016/j.heliyon.2022.e12449
- Nozdrenko D, Prylutska S, Bogutska K, et al. Effect of C₆₀ fullerene on recovery of muscle soleus in rats after atrophy induced by achillectomy. *Life*. 2022;12(3):332. doi:10.3390/life12030332
- Nozdrenko D, Matvienko T, Vygovska O, et al. Post-traumatic recovery of muscle soleus in rats is improved via synergistic effect of C₆₀ fullerene and TRPM8 agonist menthol. *Appl Nanosci*. 2021;12(3):467–478. doi:10.1007/s13204-021-01703-z

26. Nozdrenko D, Abramchuk O, Prylutska S, et al. Analysis of biomechanical parameters of muscle soleus contraction and blood biochemical parameters in rat with chronic glyphosate intoxication and therapeutic use of C₆₀ fullerene. *Int J Mol Sci*. 2021;22(9):4977. doi:10.3390/ijms22094977
27. Omelchuk O, Prylutska S, Nozdrenko D, et al. C₆₀ fullerene attenuates the signs of acute renal failure in rats under rhabdomyolysis due to inhibition of oxidative stress. *Ukr Biochem J*. 2023;95(5):61–75. doi:10.15407/ubj95.05.061
28. Prylutska SV, Matyshevska OP, Grynyuk II, et al. Biological effects of C 60 fullerenes in vitro and in a model system. *Mol Cryst Liq Cryst*. 2007;468(1):265–274. doi:10.1080/15421400701230105
29. Prilutski Y, Durov SS, Yashchuk VN, et al. Theoretical predictions and experimental studies of self-organized C₆₀ nanoparticles in water solution and on the support. *Europ Phys J D*. 1999;9(1):341–343. doi:10.1007/s100530050452
30. Prylutska SV, Grebinyk AG, Lynchak OV, et al. In vitro and in vivo toxicity of pristine C 60 fullerene aqueous colloid solution. *Fulleren Nanotub Carbon Nanostruct*. 2019;27(9):715. doi:10.1080/1536383X.2019.1634055
31. Moussa F. [60]Fullerene and derivatives for biomedical applications. *Nanobiomaterials*. 2018;113–136.
32. Gharbi N, Pressac M, Hadchouel M, et al. [60]fullerene is a powerful antioxidant *in vivo* with no acute or subacute toxicity. *Nano Lett*. 2005;5(12):2578–2585. doi:10.1021/nl051866b
33. Eswaran SV. Water Soluble Nanocarbon Materials: a Panacea for All? *Current Sci*. 2018;114(09):1846–1850. doi:10.18520/cs/v114/i09/1846-1850
34. de Souza J, Gottfried C. Muscle injury: review of experimental models. *J Electromyogr Kinesiol*. 2013;23(6):1253–1260. doi:10.1016/j.jelekin.2013.07.009
35. Kampmann JP, Hansen JM. Glomerular filtration rate and creatinine clearance. *Br J Clin Pharmacol*. 1981;12(1):7–14. doi:10.1111/j.1365-2125.1981.tb01848.x
36. Kumar D, Bagarhatta R. Fractional excretion of sodium and its association with prognosis of decompensated heart failure patients. *J Clin Diagn Res*. 2015;9(4):OC01–OC03. doi:10.7860/JCDR/2015/11532.5736
37. Suvarna KS, Layton C, Bancroft JD. *Bancroft's Theory and Practice of Histological Techniques*. 8th ed; 2018:672.
38. Stewart IJ, Faulk TI, Sosnov JA, et al. Rhabdomyolysis among critically ill combat casualties associations with acute kidney injury and mortality. *J Trauma Acute Care Surg*. 2016;80(3):492–498. doi:10.1097/TA.0000000000000933
39. Harrois A, Libert N, Duranteau J. Acute kidney injury in trauma patients. *Curr Opin Crit Care*. 2017;23(6):447–456. doi:10.1097/MCC.0000000000000463
40. Stewart IJ, Sosnov JA, Howard JT, Chung KK. Acute kidney injury in critically injured combat veterans: a retrospective cohort study. *Am J Kidney Dis*. 2016;68(4):564–570. doi:10.1053/j.ajkd.2016.03.419
41. Eriksson M, Brattström O, Mårtensson J, et al. Acute kidney injury following severe trauma: risk factors and long-term outcome. *J Trauma Acute Care Surg*. 2015;79(3):407–412. doi:10.1097/TA.0000000000000727
42. Tarazona V, Figueiredo S, Hamada S, et al. Admission serum myoglobin and the development of acute kidney injury after major trauma. *Ann Intensive Care*. 2021;11(1):140. doi:10.1186/s13613-021-00924-3
43. Sheerin NS, Sacks SH. Leaked protein and interstitial damage in the kidney: is complement the missing link? *Clin Exp Immunol*. 2002;130(1):1–3. doi:10.1046/j.1365-2249.2002.01979.x
44. Zager A. Rhabdomyolysis and myohemoglobinuric acute renal failure. *Kidney Int*. 1996;49(2):314–326. doi:10.1038/ki.1996.48
45. Vaziri ND. Dyslipidemia of chronic renal failure: the nature, mechanisms, and potential consequences. *Am J Physiol Renal Physiol*. 2006;290(2):F262–F272. doi:10.1152/ajprenal.00099.2005
46. Talving P, Karamanos E, Skiada D, et al. Relationship of creatine kinase elevation and acute kidney injury in pediatric trauma patients. *J Trauma Acute Care Surg*. 2013;74(3):912–916. doi:10.1097/TA.0b013e318278954e
47. Perkins ZB, Haines RW, Prowle JR. Trauma-associated acute kidney injury. *Curr Opin Crit Care*. 2019;25(6):565–572. doi:10.1097/MCC.0000000000000655

International Journal of Nanomedicine

Dovepress

Publish your work in this journal

The International Journal of Nanomedicine is an international, peer-reviewed journal focusing on the application of nanotechnology in diagnostics, therapeutics, and drug delivery systems throughout the biomedical field. This journal is indexed on PubMed Central, MedLine, CAS, SciSearch®, Current Contents®/Clinical Medicine, Journal Citation Reports/Science Edition, EMBase, Scopus and the Elsevier Bibliographic databases. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <https://www.dovepress.com/international-journal-of-nanomedicine-journal>



Article

C₆₀ Fullerene Reduces the Development of Post-Traumatic Dysfunction in Rat Soleus Muscle

Yuriy Prylutskyi ¹, Dmytro Nozdrenko ¹, Olexandr Motuziuk ^{1,2}, Svitlana Prylutska ³, Kateryna Bogutska ¹ , Olga Abramchuk ², Alevtyna Morenko ², Daria Franskevych ¹ , Peter Scharff ⁴ and Uwe Ritter ^{4,*}

¹ ESC “Institute of Biology and Medicine”, Taras Shevchenko National University of Kyiv, 01601 Kyiv, Ukraine; prylut@ukr.net (Y.P.); dmytro.nozdrenko@knu.ua (D.N.); motuziuk.oleksandr@vnu.edu.ua (O.M.); bogutska_ki@knu.ua (K.B.); dashaqq@gmail.com (D.F.)

² Faculty of Biology and Forestry, Lesya Ukrainka Volyn National University, 43025 Lutsk, Ukraine; abramchuk.olga@vnu.edu.ua (O.A.); morenko.alevtyna@vnu.edu.ua (A.M.)

³ Faculty of Plant Protection, Biotechnology and Ecology, National University of Life and Environmental Science of Ukraine, 03041 Kyiv, Ukraine; psvit_1977@ukr.net

⁴ Institute of Chemistry and Biotechnology, Technical University of Ilmenau, 98693 Ilmenau, Germany; peter.scharff@tu-ilmenau.de

* Correspondence: uwe.ritter@tu-ilmenau.de

Abstract: Traumatic skeletal muscle injury is a complex pathology caused by high-energy trauma to muscle tissue. Previously, a positive effect was established when C₆₀ fullerene was administered against the background of muscle ischemia, mechanical muscle injury, and other muscle dysfunctions, which probably protected the muscle tissue from damage caused by oxidative stress. Using tensiometry and biochemical analysis, the biomechanical parameters of skeletal muscle contraction and biochemical indices of the blood of rats 15 days after traumatic injury of the soleus muscle caused by myocyte destruction by compression were studied. The intraperitoneal administration of C₆₀ fullerene aqueous solution (C₆₀FAS) in a daily dose of 1 mg/kg improved its contractile function by 28–40 ± 2% and the values of the investigated biochemical indices of the animals’ blood by 15–34 ± 2% relative to the trauma group. The obtained results indicate the potential ability of C₆₀ fullerenes, as powerful antioxidants, to reduce the development of post-traumatic dysfunction of the soleus muscle.

Keywords: soleus muscle; muscle injury; C₆₀ fullerene; biomechanical and biochemical parameters



Citation: Prylutskyi, Y.; Nozdrenko, D.; Motuziuk, O.; Prylutska, S.; Bogutska, K.; Abramchuk, O.; Morenko, A.; Franskevych, D.; Scharff, P.; Ritter, U. C₆₀ Fullerene Reduces the Development of Post-Traumatic Dysfunction in Rat Soleus Muscle. *Int. J. Mol. Sci.* **2024**, *25*, 12206. <https://doi.org/10.3390/ijms252212206>

Academic Editor: Giovanna Iezzi

Received: 24 October 2024

Revised: 11 November 2024

Accepted: 12 November 2024

Published: 14 November 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

A traumatic skeletal muscle injury is a complex pathology caused by high-energy trauma to muscle tissue. Approximately 70% of combat injuries are associated with skeletal muscle injury [1]. Muscle injuries are also a common part of sports pathologies [2]. One limitation of effective therapy for muscle injuries is the lack of a unified approach to classifying muscle injuries [3]. One of the types of mechanical muscle damage is a compression injury. Such injuries are often observed following man-made disasters, road traffic accidents, and military operations [4]. The rapid death observed in some cases is due to cardiac arrhythmia caused by high concentrations of intracellular electrolytes, such as potassium, suddenly washing over the heart. Further complications are mainly due to the development of renal failure. For the effective treatment of the damaged muscular system, the timing of the start of resuscitation procedures is critical: cell death as a result of crushing occurs within 1 h after the injury, which leads to necrosis and the subsequent destruction of the muscle, causing the release of intracellular contents into the plasma [5]. Common consequences of skeletal muscle crush syndrome include hypovolemic shock, hyperkalemia, hyperphosphatemia, hypocalcemia, metabolic acidosis, arrhythmia, and disseminated intravascular coagulation [6]. After an injury, muscle fibers can repair local

damage, but they must be fully regenerated following myofibril necrosis [7]. It has been found that several weeks of non-invasive and drug-free muscle recovery after a severe injury does not lead to significant muscle regeneration, and this process is characterized by persistent inflammation, chronic activation of profibrotic markers, and extracellular matrix [8,9]. Infiltrating inflammatory and resident stem cells are involved before the repair of damaged muscle tissue. At the same time, during an intense inflammatory process, fibroblasts remain active, whereas the reparative capacity of stem cells decreases [10]. The excessive concentration of free radicals due to the inflammatory process has a detrimental effect on the regeneration processes [11]. Therefore, anti-inflammatory agents are widely used to treat muscle injuries [12], although they often lose their effectiveness due to numerous side effects [13]. For example, the immunosuppressive drug FK506 has improved the recovery of maximal muscle strength in the early phase of posttraumatic regeneration [14]. Using the antihypertensive drug Losartan reduced fibrosis and improved the regeneration of skeletal muscle injuries [15]. However, no improvement in muscle regeneration was observed in severe injuries, and the drug even negatively affected muscle function. In the absence of sufficient regeneration, the increased amount of connective tissue impairs the ability of the injured muscle to generate force, leading to decreased muscle function and impaired correction of accurate positioning. A positive therapeutic outcome has been achieved by using drugs that reduce inflammation and thereby regulate post-traumatic muscle fibrosis, the excess of which increases muscle stiffness and the risk of re-injury [16].

Due to their nanosized (almost spherical) shape, hydrophobicity, and unique chemical structure (the presence of double electron-deficient bonds), C_{60} fullerenes penetrate cells and localize preferentially in mitochondria [17], and they exhibit a strong reducing ability and act within in vitro and in vivo systems as powerful scavengers of free radicals [18,19], the overproduction of which leads to many pathologies. This opens up a valuable opportunity for the use of these nanoantioxidants, with effects surpassing those of well-known natural antioxidants—vitamins C, E, and carotenoids [20]. In vivo models previously demonstrated that the antioxidant properties of biocompatible C_{60} fullerenes [21] increased the force response of skeletal muscles after glyphosate poisoning [22] and muscle atrophy caused by prolonged immobilization [23]. A preliminary positive effect of C_{60} fullerene on soleus muscle contraction dynamics in rats after initiating ischemic injury [24] and mechanical trauma [25] was demonstrated. Thus, it can be assumed that the powerful antioxidant properties of C_{60} fullerene [18–20], reducing the level of the inflammatory process in the injured muscle of varying severity, will influence the efficiency of its post-traumatic recovery, which was the aim of the current study within the framework of the analysis of the biomechanical parameters of soleus muscle contraction and blood biochemical indices of model animals.

2. Results and Discussion

2.1. Biomechanical Analysis

Figure 1 shows the mechanograms of 10 consecutive contractions of the soleus muscle obtained on the 15th day after the initiation of muscle injury of different degrees of severity at 5 s of non-relaxation stimulation at a frequency of 50 Hz.

The integrated muscle power decreased by $11 \pm 1\%$, $19 \pm 1\%$, and $34 \pm 2\%$ at the 1st, 2nd, and 3rd degrees of injury severity, respectively, compared to the control group, indicating incomplete regeneration of damaged muscle fibers during this period at the 2nd and 3rd degrees of injury severity. The application of C_{60} fullerene aqueous solution (C_{60} FAS) injections increased the level of integrated muscle power: it almost normalized at the first injury severity grade and differed from the control group by $10 \pm 1\%$ and $14 \pm 1\%$ at the 2nd and 3rd injury severity grades, respectively (Figure 1).

The change in the value of the minimum force of muscle contraction was the main indicator of its dysfunction in each successive contractile act [26].

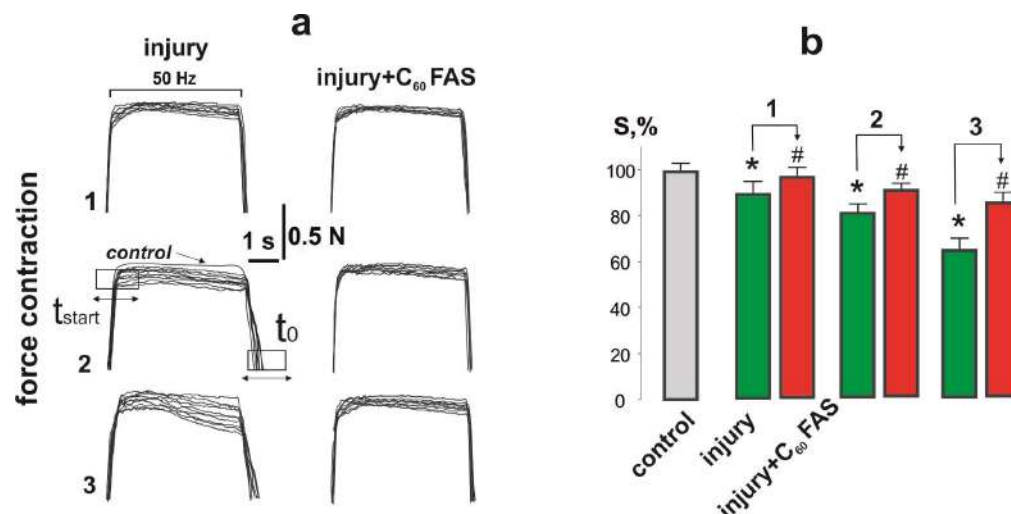


Figure 1. Force generation curves of soleus muscle contraction 15 days after its injury: mechanograms of muscle contraction (a); integrated muscle power (S; relative to the control, which was taken as 100%) (b); injury and injury + C₆₀FAS—injury group ($n = 30$) and injury group against the background of C₆₀FAS injection ($n = 30$), respectively; 1, 2, and 3—the severity of muscle injury; t_{start} —the time between the beginning of stimulation and the beginning of muscle contraction; t_0 —the time between the end of stimulation and the power curve reaching the initial level; * $p < 0.05$ relative to the control group ($n = 30$); # $p < 0.05$ relative to the injury group ($n = 30$).

The level of minimal force was 0.64 ± 0.05 , 0.59 ± 0.05 , and 0.53 ± 0.05 N at the 1st, 2nd, and 3rd degrees of injury severity, respectively, and in the control group— 0.70 ± 0.05 N. At the C₆₀FAS injections, the value of this parameter was almost normalized and was 0.68 ± 0.05 N for all degrees of injury severity (Figure 2a).

One of the main factors influencing the decrease in the biomechanical parameters described above was the increase in the stiffness components of the muscle during post-traumatic regeneration [27].

To analyze them, we measured the time between the end of the stimulation and the exit of the force curve to the initial level (Figure 2b). The t_0 time value increased from 560 ± 12 ms in the control group to 830 ± 14 , 1050 ± 9 , and 1190 ± 11 ms in injury severity 1, 2, and 3, respectively. The application of C₆₀FAS reduced this parameter to 580 ± 10 , 730 ± 8 , and 850 ± 11 ms at the 1st, 2nd, and 3rd injury severity grades, respectively. Thus, the efficacy of the C₆₀FAS therapy was $68\text{--}71 \pm 4\%$.

The formation of muscle dysfunctions is also affected by changes in nerve conduction, the disruption of which is associated with the development of the inflammatory process occurring in the post-traumatic period [28]. The change in the onset of the force response of a muscle caused by a single stimulation pool allows us to assess the level of pathological changes in the neuromuscular preparation during prolonged, static, and slow dynamic reactions of the muscular system.

The value of the t_{start} time (Figure 2c) increased from 85 ± 2 ms in the control group to 270 ± 6 , 345 ± 7 , and 430 ± 11 ms in injury severity groups 1, 2, and 3, respectively. When C₆₀FAS was applied, these values were 185 ± 12 , 281 ± 8 , and 315 ± 7 ms at the 1st, 2nd, and 3rd injury severity levels, respectively. Thus, the effect of C₆₀FAS was $21 \pm 1\%$, $18 \pm 1\%$, and $26 \pm 1\%$ at the 1st, 2nd, and 3rd injury severity levels, respectively, relative to the trauma group.

A post-traumatic increase in muscle stiffness causes increased fatigue processes in active muscles [29].

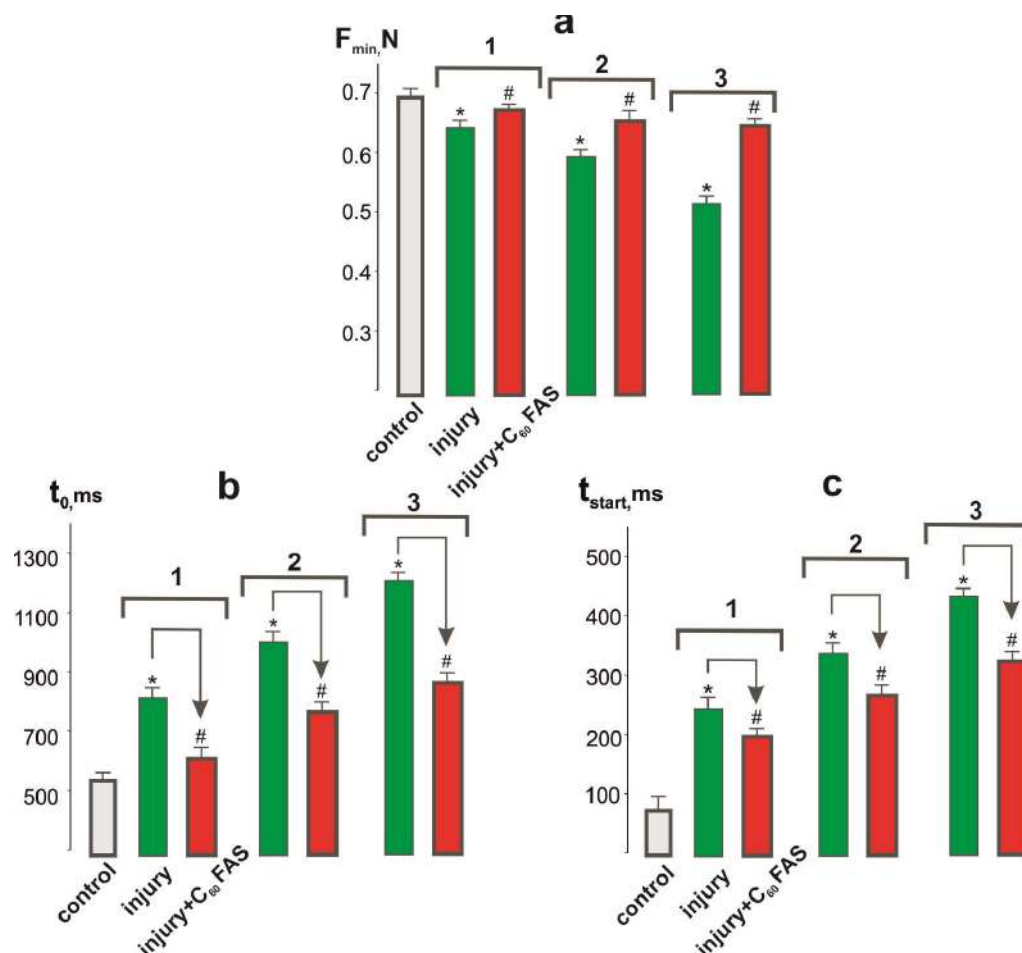


Figure 2. The biomechanical parameters of soleus muscle contraction 15 days after its injury: F_{min} —minimal force of muscle contraction (a); t_0 —time between the end of stimulation and the force curve reaching the initial level (b); t_{start} —time between the beginning of stimulation and the beginning of muscle contraction (c); injury and injury + C_{60} FAS—injury group ($n = 30$) and injury group against the background of C_{60} FAS injections ($n = 30$), respectively; 1, 2, and 3—severity of muscle injury; * $p < 0.05$ relative to the control group ($n = 30$); # $p < 0.05$ relative to the injury group ($n = 30$).

The development of muscle fatigue was assessed by calculating the time to reach 50% of the force response level when 1 Hz non-relaxation stimulation was applied (Figure 3). It should be noted that the value of this parameter in the control group was characterized by a long time interval. When registering force responses to the stimulation signal, a decrease in the force activity of the injured muscle was revealed (Figure 3)—in particular, the maximal force from $21 \pm 1\%$ at the 1st degree of injury severity to $64 \pm 3\%$ at the 3rd degree of injury severity relative to the control group (Figure 4a). The administration of C_{60} FAS increased this index by $25 \pm 1\%$, $34 \pm 2\%$, and $53 \pm 3\%$ at injury severity grades 1, 2, and 3, respectively, relative to the injury group.

The decrease in integrated muscle power (Figure 4b) was $21 \pm 1\%$, $33 \pm 2\%$, and $49 \pm 2\%$ at the 1st, 2nd, and 3rd injury severity grades, respectively, relative to the control group. C_{60} FAS injections increased this index by $18 \pm 1\%$, $23 \pm 1\%$, and $38 \pm 2\%$ at the 1st, 2nd, and 3rd injury severity levels, respectively, providing direct evidence of its positive effect on reducing connective and fibrotic tissue formation in the post-traumatic period.

The 1st, 2nd, and 3rd injury severity levels, respectively, achieved a 50% force response from the initial level, with values of 480 ± 5 , 303 ± 2 , and 202 ± 3 s (Figure 4c). The use of C_{60} FAS injections stopped the progressive fatigue processes in the injured muscle.

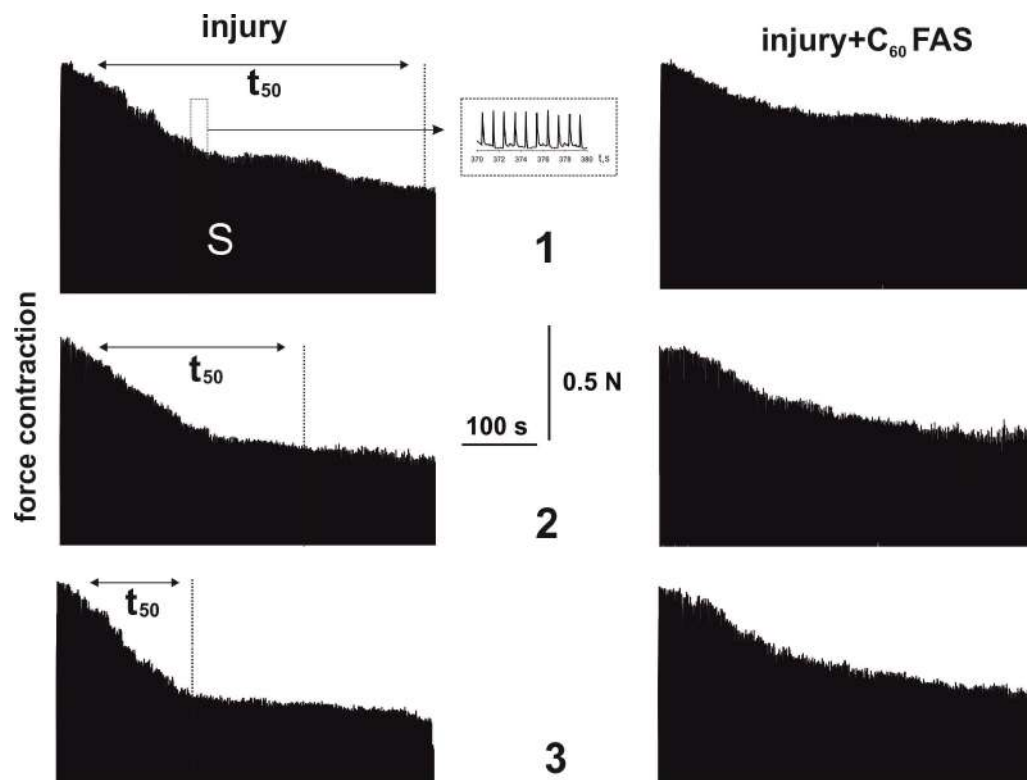


Figure 3. The mechanograms of soleus muscle contraction induced by the stimulation frequency of 1 Hz with a duration of 500 s, on the 15th day after traumatic injury initiation: injury and injury + C₆₀FAS—injury group ($n = 30$) and injury group against the background of C₆₀FAS injection ($n = 30$), respectively; 1, 2, and 3—severity of muscle injury; t_{50} —time of contraction force reduction by 50% from the initial level; S—integrated muscle power.

Summarizing the above biomechanical results, we can conclude that the administration of C₆₀FAS in a daily dose of 1 mg/kg into the injured soleus muscle during the experiment improved its contractile function by $28\text{--}40 \pm 2\%$ relative to the trauma group. This can be explained by the powerful antioxidant properties of C₆₀ fullerenes [18–20], and this conclusion is in accord with our previous results [22–25], although it requires confirmation at the biochemical level.

2.2. Biochemical Analysis

The increased stiffness components of post-traumatic muscle tissue and the insufficient regeneration of damaged muscle fibers lead to high energy expenditure during the muscle's functioning. The analysis of blood biochemical markers, in particular, creatinine and lactate levels, as well as creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) activities, allows us to evaluate the physiological changes in the muscle and the therapeutic effect of the applied drug [30]. Studies have shown that all of these biochemical markers have a pronounced tendency to increase with the increasing severity of the injury, indicating that the muscle system performs work that is super-intensive for its physiological level and subsequently develops muscle fatigue.

Changes in creatinine concentration due to the destruction of intramuscular structures allow us to assess the residual level of myocyte damage and the efficiency of the post-traumatic repair.

Figure 5 shows that the creatinine concentration increased from $54 \pm 1 \mu\text{M}$ in the control group to 177 ± 7 , 224 ± 6 , and $241 \pm 8 \mu\text{M}$ at injury severity grades 1, 2, and 3, respectively. C₆₀FAS injection resulted in its reduction by $11 \pm 1\%$, $25 \pm 1\%$, and $33 \pm 2\%$ at the 1st, 2nd, and 3rd injury severity levels, respectively, relative to the injury group. The decrease in the creatinine fraction, in our opinion, was caused by the C₆₀ fullerene protective

effect at the early stage of the pathological process by reducing inflammatory reactions and the ability to protect myocyte membranes from nonspecific free-radical destruction by absorption of reactive oxygen species (ROS) [18].

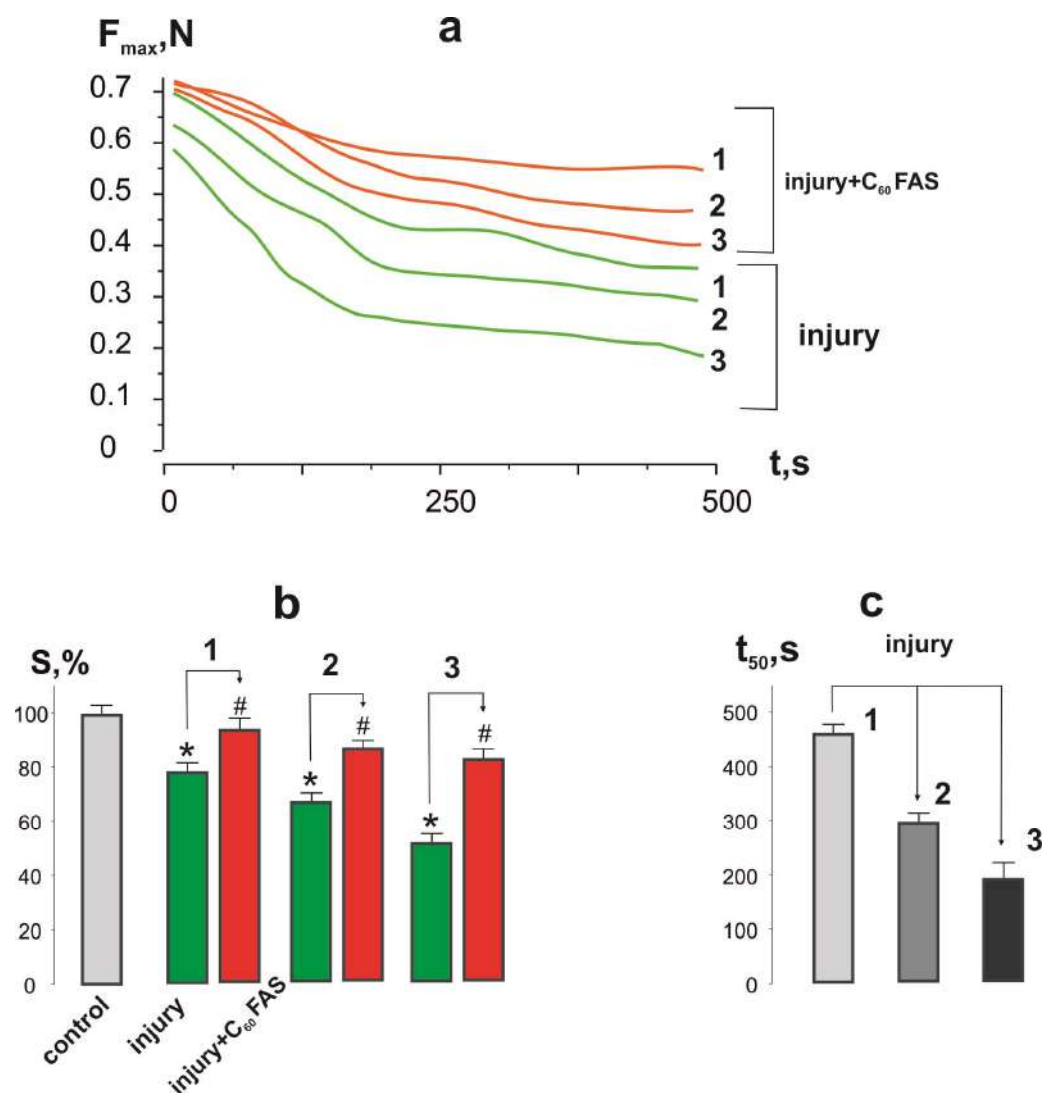


Figure 4. The biomechanical parameters of soleus muscle contraction 15 days after its injury: curves of changes in maximum contraction forces (F_{max}) during 500 s of stimulation pool (a); changes in integrated muscle power (S) during 500 s of stimulation (b); time of reduction of contraction force by 50% from the initial level (t_{50}) (c); injury and injury + C_{60} FAS—injury group ($n = 30$) and injury group against the background of C_{60} FAS injections ($n = 30$), respectively; 1, 2, and 3—severity of muscle injury; * $p < 0.05$ relative to the control group ($n = 30$); # $p < 0.05$ relative to the injury group ($n = 30$).

During the development of inflammatory reactions after the initiation of muscle injury, a significant depletion of cellular energy substances, especially ATP (adenosine triphosphate), occurs, which leads to a sharp disturbance in homeostasis and loss of ionic gradient across cell membranes. This causes the accumulation of lactate and H^+ ions and, consequently, the acidification of the pH of intra- and extracellular environments [31]. Thus, ionic changes impair the muscle's ability to respond to electrical impulses, hinder the development of excitation, and lead to a decrease in muscle strength. Consequently, the lactate level is an important marker for assessing the performance of the injured muscle.

The analysis of blood lactate content showed an increase from 10 ± 1 mM in the control group to 15 ± 1 , 17 ± 1 , and 20 ± 2 mM at the 1st, 2nd, and 3rd injury severity levels, respectively. The C_{60} FAS injection reduced this index by $12 \pm 1\%$, $19 \pm 1\%$, and

$37 \pm 2\%$ at the 1st, 2nd, and 3rd injury severity levels, respectively, relative to the trauma group (Figure 5).

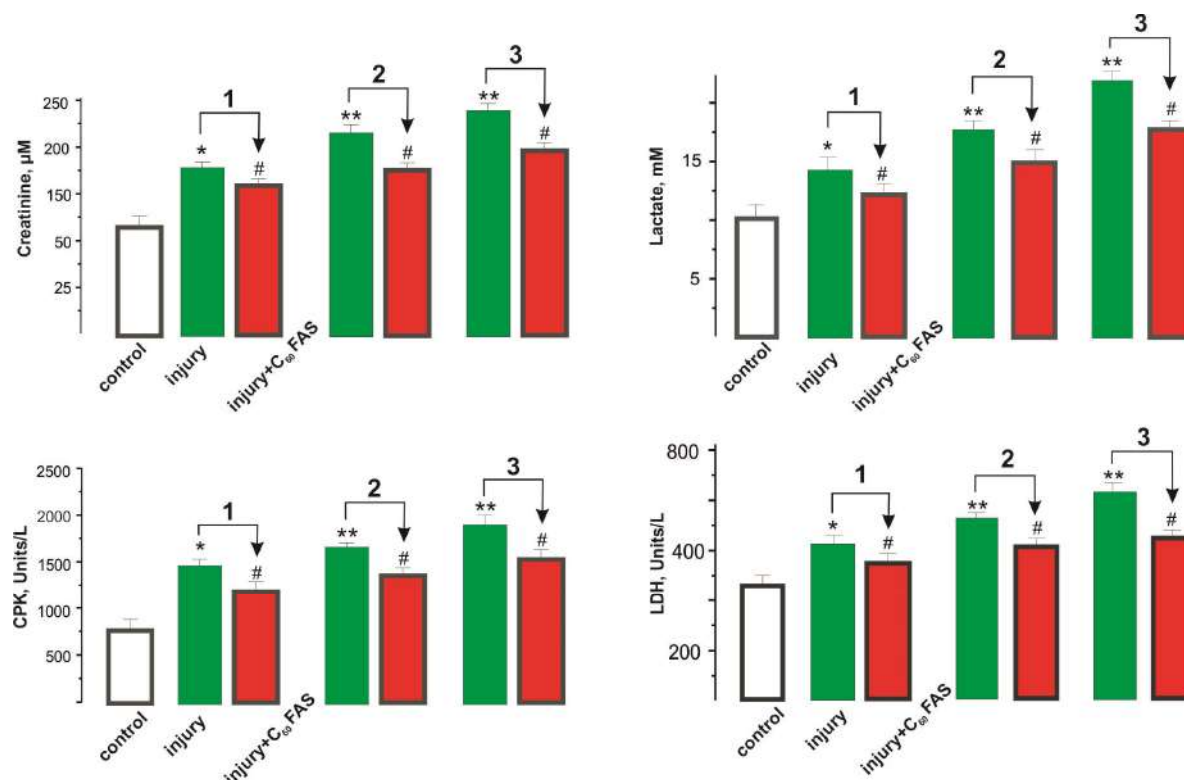


Figure 5. The biochemical indices of fatigue processes' development (the levels of creatinine and lactate and the activities of CPK and LDH in blood plasma) 15 days after soleus muscle injury: injury and injury + C₆₀FAS—the injury group ($n = 30$) and the injury group against the background of C₆₀FAS injections ($n = 30$), respectively; 1, 2, and 3—degrees of severity of muscle injury; * $p < 0.05$ relative to the control group ($n = 30$); ** $p < 0.04$ relative to the control group ($n = 30$); # $p < 0.05$ relative to the injury group ($n = 30$).

One of the known markers of muscle fatigue is a change in the activity of CPK, an enzyme from the energy supply system of skeletal muscle cells. During intensive muscle functioning, this enzyme is released from the cells and, accordingly, creates an increase in CPK activity in the blood [30].

The increase in CPK activity from 620 ± 17 Units/L in the control group to 1410 ± 23 , 1620 ± 32 , and 1840 ± 29 Units/L at the 1st, 2nd, and 3rd degrees of injury severity, respectively, indicates an increased energy load on the injured muscle due to increased connective tissue and the dysfunction of post-traumatic muscle fibers [32]. CPK activity decreased by $24\text{--}28 \pm 2\%$ relative to the trauma group when injected with C₆₀FAS (Figure 5).

The change in the activity of LDH, an enzyme that catalyzes the oxidation of lactic acid, allowed us to assess the performance of the injured muscle after its prolonged stimulation [31].

The increase of LDH activity from 310 ± 9 Units/L to 390 ± 13 , 413 ± 15 , and 493 ± 14 Units/L at the 1st, 2nd, and 3rd degrees of injury severity, respectively, indicates the development of significant dysfunctions of the neuromuscular apparatus and, as a consequence, fatigue processes. The C₆₀FAS injection decreased LDH activity by $14 \pm 1\%$, $21 \pm 1\%$, and $37 \pm 2\%$ at the 1st, 2nd, and 3rd injury severity levels, respectively, relative to the injury group (Figure 5).

Summarizing the above biochemical results, we can conclude that the administration of C₆₀FAS in a daily dose of 1 mg/kg into the injured soleus muscle during the experiment improved the values of the investigated biochemical indices of the animals' blood by

15–34 ± 2% relative to the trauma group. This can be explained as follows: C₆₀ fullerenes, as powerful antioxidants, can effectively inactivate ROS [18–20], protecting myocytes from damage and reducing the inflammation of the injured muscle, thereby reducing fibrosis [33] and thus preventing the development of post-traumatic soleus muscle dysfunction, which requires further preclinical testing.

3. Materials and Methods

3.1. Preparation of C₆₀FAS

C₆₀FAS was prepared according to the method [34]. Briefly, we used a saturated solution of C₆₀ fullerene (purity > 99.99%) in toluene with a C₆₀ molecule concentration corresponding to maximum solubility near 2.9 mg/mL and the same amount of distilled water in an open beaker. The two phases formed were treated in an ultrasonic bath (8 Hz, 8 h). The procedure was continued until the toluene had completely evaporated and the water phase became yellow-colored. Filtration of the aqueous solution (the pore size of the filter was smaller than 2 µm) allowed the separation of the product from undissolved C₆₀ fullerene. The resulting C₆₀FAS, with a maximum concentration of 0.15 mg/mL, is a typical colloid containing both single C₆₀ molecules (~0.7 nm) and their nanoaggregates up to ~100 nm in size, and its zeta potential value was -25 ± 2 mV [35]. Moreover, C₆₀FAS was stable for 12–18 months at a storage temperature of +4–20 °C.

3.2. In Vivo Experiments

The experiments were performed on male Wistar rats aged 1 to 1.5 months (at the end of the experiment). The rats were kept under controlled environmental conditions (21 °C, 12 h light–12 h dark cycle) with free access to water and standard rodent chow. The study protocol (No. 9, dated 4 September 2023) was approved by the Bioethics Committee of the ESC “Institute of Biology and Medicine” of Taras Shevchenko National University of Kyiv in accordance with the rules of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and the norms of biomedical ethics in accordance with the Law of Ukraine No. 3447-IV of 21.02.2006, Kyiv, “On the Protection of Animals from Cruelty” during biomedical research.

Muscle injury was induced by compressing the soleus muscle for 1 (the 1st degree of injury severity), 2 (the 2nd degree of injury severity), and 3 (the 3rd degree of injury severity) min with a clamp under a pressure of 3.5 kg/cm² [36,37]: We made small incisions in the skin of laboratory animals, where the branches of the crusher device were placed to prevent mechanical impact on the nearby gastrocnemius muscle. This process was controlled visually using a FIM-17 fiber optic microscope (Grandway Telecom Tech., Shanghai, China).

The applied crush syndrome leads to the destruction of myocytes, specifically the release of their components (creatine kinase, lactic acid, and myoglobin) into the extracellular environment, which serves as a marker of the severity of muscle injury. The 1st degree of severity of muscle injury is characterized by the manifestation of numerous local intramuscular hematomas and ruptures of individual muscle cells without damage to the integrity of the muscle membranes; a characteristic manifestation of the 2nd degree of severity of muscle injury is ruptures of the fascial structures of the muscle, damage to the muscle innervation, inflammation and swelling of the muscle, damage to blood vessels, and, as a result, large superficial and intramuscular hematomas; the 3rd degree of severity of muscle injury is characterized by ruptures of muscle sheaths, fascia and subfascial structures, muscle tendons, blood vessels, nerves, auxiliary apparatus of the muscle, and tissues adjacent to the muscle [36–39].

Experimental animals were divided into the following groups: control ($n = 30$), model injury of the 1st, 2nd, and 3rd severity degrees ($n = 30$), and model injury of the 1st, 2nd, and 3rd severity after administration of C₆₀FAS ($n = 30$). C₆₀FAS was administered intraperitoneally daily throughout the experiment, starting on the first day immediately after the initiation of muscle injury.

Previously we investigated the impact of different doses of C₆₀FAS (0.5, 1, 1.5, and 2 mg/kg) on various in vivo models of muscle pathologies [22–25] and found that the 1 mg/kg of C₆₀FAS dose demonstrated the highest efficacy in the therapy. Therefore, this dose was chosen for these experiments. In addition, it is significantly lower than the LD₅₀ value (lethal dose, 50%), which was 600 mg/kg in the case of oral administration to rats [18] and 721 mg/kg in the case of intraperitoneal administration to mice [35]. It is also important to note that after intravenous administration to mice, C₆₀ fullerenes accumulate predominantly in the blood, spleen, stomach, and liver and are excreted from the body within 72 h, mainly with urine [40].

All experimental studies were conducted during a 15-day post-injury period. This is due to the fact that the main processes of repairing damaged muscles in the conditions of their natural post-traumatic recovery last for 12–15 days [41].

3.3. Materials for Biomechanical Analysis

Animals were anesthetized (to study the functional activity of the soleus muscle) by intraperitoneal injection of nembutal (40 mg/kg). The soleus muscle of the rat was freed from the surrounding tissues. Its tendon part was cut across the distal part of the lumbar spine. For modulated stimulation of efferents in the L7-S1 segments, the ventral roots were cut at the sites of their exit from the spinal cord. Electrical pulses performed a stimulation of soleus muscle efferents with a duration of 2 ms, generated by a pulse generator through platinum electrodes. The current strength at which the muscle started to contract was considered the threshold, and further stimulation was performed at a strength of 1.3–1.4 of that threshold. The external load on the soleus muscle was controlled using a system of mechanical stimulators. To record the force of skeletal muscle contraction, an original strain gauge setup consisting of force and length sensors, a synchronous pulse generator, and a temperature control system was used [22–25].

When analyzing the contractile activity of the soleus muscle, the following basic biomechanical parameters were evaluated [22–25]:

- integrated muscle power—calculated area (S) under the power curve using Origin 9.4 software, which is an indicator of the overall performance of the muscle at applied stimulation pools;
- $F_{\min}$ and $F_{\max}$ —minimum and maximum forces of contraction;
- t_0 —the time between the end of stimulation and the force curve reaching the initial level;
- t_{start} —the time between the beginning of stimulation and the beginning of muscle contraction;
- t_{50} —time of contraction force reduction by 50% of the initial level.

3.4. Materials for Biochemical Analysis

Blood biochemical indices of experimental animals, such as creatinine and lactate levels and activities of CPK and LDH, which are the most commonly used clinical markers of muscle injury [42], were determined using the following diagnostic equipment: biochemical analyzers RNL-200 and JN-1101-TR2 (Amsterdam, The Netherlands).

3.5. Statistical Analysis

The statistical evaluation of the experimental results was performed using the procedure of variance analysis (ANOVA) with mixed design. Two between-group factors were supposed: (1) injury (three levels—the 1st, 2nd, and 3rd degrees of severity); (2) C₆₀FAS treatment (two levels—no and use of C₆₀FAS). The Shapiro–Wilk *W*-test was used to test for normality. Levene’s test was used to assess the equality of variances across groups. Multiple pairwise comparisons between different groups and conditions were performed by the Bonferroni post-hoc test. The differences between the groups were considered significant at $p < 0.05$. Each of the experimental force curves was the result of averaging 10 similar tests. Each biochemical measurement was carried out at least three times. The statistical

evaluation was performed using the software package Statistica 8.0 (Dell Technologies Inc., Round Rock, TX, USA).

4. Conclusions

To date, the powerful antioxidant properties of C₆₀ fullerenes have been widely used in biomedicine. Due to the negative effects of ROS in oxidative stress processes, antioxidants are required to protect injured muscles. Here, the effect of water-soluble C₆₀ fullerenes (daily intramuscular dose of 1 mg/kg) on the process of the restoration of contractile activity of rat skeletal muscle on the 15th day after the initiation of injury of varying severity was studied for the first time. The revealed improvement in the dynamics of the soleus muscle functioning by $28\text{--}40 \pm 2\%$ and biochemical indices of rat blood by $15\text{--}34 \pm 2\%$ opens the prospect of applying C₆₀ fullerenes as potential nanoagents that are able to effectively correct the pathological state of skeletal muscle arising from its mechanical trauma.

Author Contributions: Study design, investigation, data analysis, manuscript drafting, D.N., O.M., S.P., K.B., O.A., A.M., D.F. and P.S.; conceptualization, methodology, contribution to the interpretation of the results, writing—original draft preparation, revision of the manuscript, approval of the final submission, writing—review and editing, Y.P. and U.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Research Foundation of Ukraine (grant number 2022.01/0004).

Institutional Review Board Statement: The study protocol (No. 9, dated 4 September 2023) was approved by the Bioethics Committee of the ESC “Institute of Biology and Medicine” of Taras Shevchenko National University of Kyiv in accordance with the rules of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and the norms of biomedical ethics in accordance with the Law of Ukraine No. 3447-IV of 21 February 2006, Kyiv, “On the Protection of Animals from Cruelty” during biomedical research.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Covey, D.C. Combat orthopaedics: A view from the trenches. *J. Am. Acad. Orthop. Surg.* **2006**, *14*, S10–S17. [[CrossRef](#)] [[PubMed](#)]
2. Kibler, W.B. Clinical aspects of muscle injury. *Med. Sci. Sports Exerc.* **1990**, *22*, 450–452. [[CrossRef](#)] [[PubMed](#)]
3. Hamilton, B.; Valle, X.; Rodas, G.; Til, L.; Grive, R.P.; Gutierrez Rincon, J.A.; Tol, J.L. Classification and grading of muscle injuries: A narrative review. *Br. J. Sports Med.* **2015**, *49*, 306. [[CrossRef](#)] [[PubMed](#)]
4. VandenBerg, S.L.; Davidson, S.B. Preparation for mass casualty incidents. *Crit. Care Nurs. Clin. North. Am.* **2015**, *27*, 157–166. [[CrossRef](#)] [[PubMed](#)]
5. Lovallo, E.; Koyfman, A.; Foran, M. Crush syndrome le syndrome de compression. *Af. J. Emerg. Med.* **2012**, *2*, 117–123. [[CrossRef](#)]
6. Gibney, R.T.N.; Sever, M.S.; Vanholder, R.C. Disaster nephrology: Crush injury and beyond. *Kidney Int.* **2014**, *85*, 1049–1057. [[CrossRef](#)]
7. Edouard, P.; Reurink, G.; Mackey, A.L.; Lieber, R.L.; Pizzari, T.; Järvinen, T.A.H.; Gronwald, T.; Hollander, K. Traumatic muscle injury. *Nat. Rev. Dis. Primers* **2023**, *9*, 56. [[CrossRef](#)]
8. Karalaki, M.; Fili, S.; Philippou, A.; Koutsilieris, M. Muscle regeneration: Cellular and molecular events. *In Vivo* **2009**, *23*, 779–796.
9. Forcina, L.; Cosentino, M.; Musarò, A. Mechanisms Regulating Muscle Regeneration: Insights into the Interrelated and Time-Dependent Phases of Tissue Healing. *Cells* **2020**, *9*, 1297. [[CrossRef](#)]
10. Mann, C.J.; Perdiguero, E.; Kharraz, Y.; Aguilar, S.; Pessina, P.; Serrano, A.L.; Muñoz-Cánoves, P. Aberrant repair and fibrosis development in skeletal muscle. *Skelet. Muscle* **2011**, *1*, 21. [[CrossRef](#)]
11. Wagers, A.J.; Conboy, I.M. Cellular and molecular signatures of muscle regeneration: Current concepts and controversies in adult myogenesis. *Cell* **2005**, *122*, 659–667. [[CrossRef](#)] [[PubMed](#)]
12. Duchesne, E.; Dufresne, S.S.; Dumont, N.A. Impact of Inflammation and Anti-inflammatory Modalities on Skeletal Muscle Healing: From Fundamental Research to the Clinic. *Phys. Ther.* **2017**, *97*, 807–817. [[CrossRef](#)] [[PubMed](#)]

13. Perandini, L.A.; Chimin, P.; da Silva Lutkemeyer, D.; Saraiva Câmara, N.O. Chronic inflammation in skeletal muscle impairs satellite cells function during regeneration: Can physical exercise restore the satellite cell niche? *FEBS J.* **2018**, *285*, 1973–1984. [[CrossRef](#)] [[PubMed](#)]
14. Aydin, M.A.; Urbanchek, M.G.; Kuzon, W.M. Improved early muscle recovery using FK506 in a rat nerve-repair model. *J. Reconstr. Microsurg.* **2004**, *20*, 183–192. [[CrossRef](#)]
15. Garg, K.; Corona, B.T.; Walters, T.J. Losartan administration reduces fibrosis but hinders functional recovery after volumetric muscle loss injury. *J. Appl. Physiol.* **2014**, *17*, 1120–1131. [[CrossRef](#)]
16. Hubbert, K.; Clement, R. Losartan: A Novel Treatment for Acute Skeletal Muscle Injury. *JBJS J. Orthop. Phys. Assist.* **2021**, *9*, e20. [[CrossRef](#)]
17. Foley, S.; Crowley, C.; Smahi, M.; Bonfils, C.; Erlanger, B.F.; Seta, P.; Larroque, C. Cellular localisation of a water-soluble fullerene derivative. *Biochem. Biophys. Res. Commun.* **2002**, *294*, 116–119. [[CrossRef](#)]
18. Gharbi, M.; Pressac, M.; Hadchouel, M.; Szwarc, H.; Wilson, S.R.; Moussa, F. [60]fullerene is a powerful antioxidant in vivo with no acute or subacute toxicity. *Nano Lett.* **2005**, *5*, 2578–2585. [[CrossRef](#)]
19. Goodarzi, S.; Da Ros, T.; Conde, J.; Sefat, F.; Mozafari, M. Fullerene: Biomedical engineers get to revisit an old friend. *Mater. Today* **2017**, *20*, 460–480. [[CrossRef](#)]
20. Eswaran, S.V. Water Soluble Nanocarbon Materials: A Panacea for All? *Current Sci.* **2018**, *114*, 1846–1850. [[CrossRef](#)]
21. Halenova, T.; Raksha, N.; Savchuk, O.; Ostapchenko, L.I.; Prylutsky, Y.I.; Ritter, U.; Scharff, P. Evaluation of the biocompatibility of water-soluble pristine C₆₀ fullerenes in rabbit. *BioNanoScience* **2020**, *10*, 721–730. [[CrossRef](#)]
22. Nozdrenko, D.; Abramchuk, O.; Prylutska, S.; Vygovska, O.; Soroca, V.; Bogutska, K.; Khrapatyi, S.; Prylutsky, Y.; Scharff, P.; Ritter, U. Analysis of Biomechanical Parameters of *Muscle Soleus* Contraction and Blood Biochemical Parameters in Rat with Chronic Glyphosate Intoxication and Therapeutic Use of C₆₀ Fullerene. *Int. J. Mol. Sci.* **2021**, *22*, 4977. [[CrossRef](#)] [[PubMed](#)]
23. Nozdrenko, D.; Prylutska, S.; Bogutska, K.; Nurishchenko, N.Y.; Abramchuk, O.; Motuziuk, O.; Prylutsky, Y.; Scharff, P.; Ritter, U. Effect of C₆₀ Fullerene on Recovery of *Muscle Soleus* in Rats after Atrophy Induced by Achillotenotomy. *Life* **2022**, *12*, 332. [[CrossRef](#)] [[PubMed](#)]
24. Nozdrenko, D.; Matvienko, T.; Vygovska, O.; Bogutska, K.; Motuziuk, O.; Nurishchenko, N.Y.; Prylutsky, Y.; Scharff, P.; Ritter, U. Protective Effect of Water-Soluble C₆₀ Fullerene Nanoparticles on the Ischemia-Reperfusion Injury of the *Muscle Soleus* in Rats. *Int. J. Mol. Sci.* **2021**, *22*, 6812. [[CrossRef](#)]
25. Nozdrenko, D.; Matvienko, T.; Vygovska, O.; Soroca, V.; Bogutska, K.; Zholos, O.; Scharff, P.; Ritter, U.; Prylutsky, Y. Post-traumatic recovery of *muscle soleus* in rats is improved via synergistic effect of C₆₀ fullerene and TRPM8 agonist menthol. *Appl. Nanosci.* **2022**, *12*, 467–478. [[CrossRef](#)]
26. Kostyukov, A.I.; Gorkovenko, A.V.; Kulyk, Y.A.; Lehedza, O.V.; Shushuiev, D.I.; Zasada, M.; Strafun, S.S. Central Commands to the Elbow and Shoulder Muscles During Circular Planar Movements of Hand With Simultaneous Generation of Tangential Forces. *Front. Physiol.* **2022**, *13*, 864404. [[CrossRef](#)]
27. Chargé, S.B.P.; Rudnicki, M.A. Cellular and molecular regulation of muscle regeneration. *Physiol. Rev.* **2004**, *84*, 209–238. [[CrossRef](#)]
28. Bedair, H.; Liu, T.T.; Kaar, J.L.; Badlani, S.; Russell, A.J.; Li, Y.; Huard, J. Matrix metalloproteinase-1 therapy improves muscle healing. *J. Appl. Physiol.* **2007**, *102*, 2338–2345. [[CrossRef](#)]
29. Whiteside, L.A. Surgical technique: Gluteus maximus and tensor fascia lata transfer for primary deficiency of the abductors of the hip. *Clin. Orthop. Relat. Res.* **2014**, *472*, 645–653. [[CrossRef](#)]
30. Arenas, J.; Diaz, V.; Liras, G.; Gutierrez, E.; Santos, I.; Martinez, A.; Culebras, J.M. Activities of creatine kinase and its isoenzymes in serum in various skeletal muscle disorders. *Clin. Chem.* **1988**, *34*, 2460–2462. [[CrossRef](#)]
31. Hagberg, H. Intracellular pH during ischemia in skeletal muscle: Relationship to membrane potential, extracellular pH, tissue lactic acid and ATP. *Pflügers Arch.* **1985**, *404*, 342–347. [[CrossRef](#)] [[PubMed](#)]
32. Kazezian, Z.; Bul, A.M.J. A review of the biomarkers and in vivo models for the diagnosis and treatment of heterotopic ossification following blast and trauma-induced injuries. *Bone* **2021**, *143*, 115765. [[CrossRef](#)] [[PubMed](#)]
33. Ohmae, S.; Akazawa, S.; Takahashi, T.; Izumo, T.; Rogi, T.; Nakai, M. Quercetin attenuates adipogenesis and fibrosis in human skeletal muscle. *Biochem. Biophys. Res. Commun.* **2022**, *615*, 24–30. [[CrossRef](#)] [[PubMed](#)]
34. Scharff, P.; Carta-Abelmann, L.; Siegmund, C.; Matyshevska, O.P.; Prylutska, S.V.; Koval, T.V.; Golub, A.A.; Yashchuk, V.M.; Kushnir, K.M.; Prylutsky, Y.I. Effect of X-ray and UV irradiation of the C₆₀ fullerene aqueous solution on biological samples. *Carbon* **2004**, *42*, 1199–1201. [[CrossRef](#)]
35. Prylutska, S.V.; Grebinyk, A.G.; Lynchak, O.V.; Byelinska, I.; Cherepanov, V.; Täuscher, E.; Matyshevska, O.; Prylutsky, Y.I.; Rybalchenko, V.; Ritter, U.; et al. In vitro and in vivo toxicity of pristine C₆₀ fullerene aqueous colloid solution. *Fuller. Nanotub. Carbon Nanostructures* **2019**, *27*, 715–728. [[CrossRef](#)]
36. Chen, X.; Liu, Y.; Xu, W.; Qin, T.; Zhao, L.; Liu, S.; Zhang, Y.; Tan, H.; Zhou, Y. Experimental study on establishment of a simple model of rats crush injury-crush syndrome. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi.* **2013**, *27*, 77–82.
37. Li, D.; Chen, Y.; Yang, B.; Li, W.; Xu, Y.; Wang, X.; Teng, Y.; Luo, Y.; Liu, C.; Shi, J.; et al. Breaking new ground: Standardizing rat models for crush syndrome investigations. *Shock* **2024**, *61*, 274–282. [[CrossRef](#)]
38. De Souza, J.; Gottfried, C. Muscle injury: Review of experimental models. *J. Electromyogr. Kinesiol.* **2013**, *23*, 1253–1260. [[CrossRef](#)]

39. Liu, Y.; Yu, M.; Chen, L.; Liu, J.; Li, X.; Zhang, C.; Xiang, X.; Li, X.; Lv, Q. Systemic Review of Animal Models Used in the Study of Crush Syndrome. *Shock* **2022**, *57*, 469–478. [[CrossRef](#)]
40. Nikolic, N.; Vranjes-Ethuric, S.; Jankovic, D.; Ethokic, D.; Mirkovic, M.; Bibic, N.; Trajkovic, V. Preparation and biodistribution of radiolabeled fullerene C₆₀ nanocrystals. *Nanotechnol.* **2009**, *20*, 385102. [[CrossRef](#)]
41. Best, T.M.; Hunter, K.D. Muscle injury and repair. *Phys. Med. Rehabil. Clin. N. Am.* **2000**, *11*, 251–266. [[CrossRef](#)] [[PubMed](#)]
42. Brancaccio, P.; Lippi, G.; Maffulli, N. Biochemical markers of muscular damage. *Clin. Chem. Lab. Med.* **2010**, *48*, 757–767. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

C₆₀ fullerene improves the contractile activity of the injured rat *muscle gastrocnemius*

Yuriy Prylutsky¹ , Dmytro Nozdrenko¹ , Olexandr Motuziuk^{1,2} , Svitlana Prylutska³ , Natalia Nurishchenko¹ , Daria Franskevych¹ , Vasil Soroca¹ , Vsevolod Cherepanov⁴ , Ihor Kalinin³ , Olha Korzhyk²  and Uwe Ritter^{5,*} 

¹ ESC 'Institute of Biology and Medicine', Taras Shevchenko National University of Kyiv, 01601 Kyiv, Ukraine

² Faculty of Biology and Forestry, Lesya Ukrainka Volyn National University, 43025 Lutsk, Ukraine

³ Faculty of Plant Protection, Biotechnology and Ecology and Faculty of Veterinary Medicine, National University of Life and Environmental Science of Ukraine, 03041 Kyiv, Ukraine

⁴ Institute of Physics, NAS of Ukraine, 03028 Kyiv, Ukraine

⁵ Institute of Chemistry and Biotechnology, Technical University of Ilmenau, 98693 Ilmenau, Germany

E-mail: uwe.ritter@tu-ilmenau.de

Received 22 August 2024, revised 11 December 2024

Accepted for publication 23 December 2024

Published 27 January 2025



Abstract

The powerful antioxidant properties of C₆₀ fullerenes have been widely used in biomedical nanotechnology. Owing to the negative effects of free radicals in oxidative stress processes, antioxidants are required to protect injured muscles. Here, the effect of water-soluble C₆₀ fullerenes (daily oral dose 1 mg kg⁻¹) on the process of restoration of contractile activity of skeletal muscle of rats (*muscle gastrocnemius*) 15 d after the initiation of open trauma of different severity was studied for the first time. The structural organization of C₆₀ fullerene nanoparticles in aqueous solution was analyzed by dynamic light scattering and atomic force microscopy techniques. Such biomechanical parameters of *muscle gastrocnemius* contraction as integrated muscle power, levels of generation of its maximum and minimum force, and time interval until reaching 50% of the level of force response of the muscle were analyzed. Such biochemical indices as concentrations of c-reactive protein, creatinine, and lactate in the rat blood, as well as indices of pro- and antioxidant balance (activities of superoxide dismutase and catalase, the concentration of reduced glutathione) in the blood and muscle tissue of experimental animals, were investigated. It was found that application of water-soluble C₆₀ fullerenes statistically significantly improves biomechanical parameters of contraction of injured *muscle gastrocnemius* at the level of 30–45 ± 3%, which is confirmed by normalization of biochemical indices in the blood and muscle tissue of rats at the level of 35–50 ± 3% and 20–37 ± 3%, correspondingly, relative to the open injury group. These findings open the possibility of using C₆₀ fullerenes as potential therapeutic nanoagents capable of correcting pathological states of the muscular system during the physiological repair of open injuries.

* Author to whom any correspondence should be addressed.



Original content from this work may be used under the terms of the [Creative Commons Attribution 4.0 licence](https://creativecommons.org/licenses/by/4.0/). Any further distribution of this work must maintain attribution to the author(s) and the title of the work, journal citation and DOI.

Keywords: C₆₀ fullerene, *muscle gastrocnemius*, open muscle injury, biomechanical parameters of muscle contraction, blood and muscle tissue biochemical indicators, dynamic light scattering, atomic force microscopy

1. Introduction

Open trauma is one of the most common skeletal muscle injuries among both civilians and military personnel [1, 2], and the complications caused by such trauma can be significant [3, 4]. With timely and appropriate treatment ranging from simple closure of injured areas to complex tissue transfer, the threat of limb loss can be prevented [5]. Differences in the severity of the injury sustained and the affected muscle group, as well as the non-specificity of symptoms, complicate research to find effective treatments. In this case, it is important to understand the cellular processes involved in muscle healing. Acute skeletal muscle injury is known to result in fiber destruction, oxidative stress, and inflammation [6, 7]. The complication of healing is often associated with secondary damage to surrounding muscle tissues with the development of inflammatory reactions. The inflammatory reaction depends on two factors, namely the degree of actual physical injury and the degree of muscle vascularization at the time of injury. Studies [8, 9] have shown that the use of various anti-inflammatory treatments is not effective enough to promote the healing of open muscle injuries. Therefore, there is an urgent need to find new techniques and drugs that improve the healing processes of injured muscles [6, 10, 11].

Antioxidant therapy is quite effective in reducing the level of inflammation in muscle injuries [10, 12]. For example, the proanthocyanidin oligomer resveratrol, an antioxidant derived from grape seeds, reduces inflammation in injured muscles [13]. It also improved skeletal muscle regeneration after injury compared to conventional treatment with non-steroidal anti-inflammatory drugs. Melittin, an antioxidant isolated from bee venom, enhances the expression of muscle regeneration factors in a mouse model of skeletal muscle injury [14].

Due to the presence of double electron-deficient chemical bonds in the structure, C₆₀ fullerene, as a third allotropic form of carbon, exhibits a strong reducing ability and acts in *in vitro* and *in vivo* systems as a powerful scavenger of free radicals [15–17], the overproduction of which leads to many pathologies. This opens up a real opportunity for the practical use of this nanoantioxidant, with effects surpassing those of well-known natural antioxidants—vitamins C, E, and carotenoids [18] as well as *N*-acetylcysteine and β -alanine [19], which are often used in sports medicine. So, it has been shown that *in vivo* application of an aqueous solution of C₆₀ fullerene leads to significant positive therapeutic effects in muscle pathologies of different origins [20–23]. Based on the above data, the present work aimed to investigate the effect of C₆₀ fullerenes on the process of recovery of contractile activity of rat skeletal muscle (*muscle gastrocnemius*) 15 d after initiation of open trauma of different severity.

2. Materials and methods

2.1. Preparation of water-soluble C₆₀ fullerenes and characterization

A method, based on the transfer of C₆₀ molecules from toluene solution to water followed by sonication [24], has been used to preparation of water-soluble C₆₀ fullerenes. The resulting fluid at a maximum C₆₀ fullerene concentration of 0.15 mg ml^{−1} remains stable for 18 months at a temperature of +4 °C–25 °C.

Measurements of the size distribution for C₆₀ fullerenes in aqueous solution and the zeta potential value for them were performed by dynamic light scattering (DLS) on a NanoBrook Omni (Brookhaven, NY, USA) at *T* = 298 K. A DLS instrument equipped with a He–Ne laser (max 5 mW) operating at the wavelength of 512 nm was used. The results were evaluated using the Smoluchowski approximation, which is known to be rigorously valid only for spherical-like particles.

Atomic force microscopy (AFM; ‘Solver ProM’ system in tapping mode) was used to study the structural organization of C₆₀ fullerene in an aqueous solution. To prepare the sample, a drop of water-soluble C₆₀ fullerenes was applied to an atomically smooth surface of the mica substrate (SPI Supplies, Grade V1 Muscovite). The visible water evaporation process was monitored using an optical microscope built into the ‘Solver ProM’ AFM system. The sample was further dried under ambient conditions for 24 h before AFM measurements.

2.2. In vivo study

The experiments were conducted on 3-month-old male Wistar rats weighing 170 ± 5 g. All procedures complied with the ARRIVE guidelines. The use of the laboratory animals was approved by the Biomedical Ethics Committee of the ESC ‘Institute of Biology and Medicine’ of Taras Shevchenko National University of Kyiv (protocol No. 9 dated 4 September 2023) and performed under the ‘European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes’ (Strasbourg, 1986) and Article 26 of the Law of Ukraine ‘On the Protection of Animals from Cruelty’ (No. 3447-IV, 21.02.2006), as well as European Union Directive of 22 September 2010 (2010/63/EU) for the protection of animals used for scientific purposes.

The rats were randomly divided into the following experimental groups: control group (*n* = 10); open injury (non-treated) group 1 (*n* = 10), 2 (*n* = 10), and 3 (*n* = 10) of the 1st, 2nd, and 3rd degree of severity, respectively; open injury + C₆₀ (treated) group 1 (*n* = 10), 2 (*n* = 10) and 3 (*n* = 10) of the 1st, 2nd and 3rd degree of severity, respectively (oral daily use of water-soluble C₆₀ fullerenes in a

dose of 1 mg kg^{-1} animal body weight after initiation of injury).

Previously we investigated the impact of different doses of C_{60}FAS (0.5 , 1 , 1.5 , and 2 mg kg^{-1}) on various *in vivo* models of muscle pathologies [20–23] and found that the 1 mg kg^{-1} of C_{60}FAS dose demonstrated the high efficacy in the therapy. Therefore, this dose was chosen in the experiments. In addition, the total dose of 15 mg kg^{-1} in the experiment is significantly lower than the LD_{50} value (lethal dose, 50%), which was 600 mg kg^{-1} in the case of oral administration to rats [15] and is therefore safe for bioapplication. It is also important to note that after intravenous administration to mice, the radiolabeled C_{60} fullerenes accumulate predominantly in the blood, spleen, stomach, and liver and are excreted within 72 h, mainly with urine [25, 26].

All experimental studies were conducted on a 16 d post-injury period. This is because the main processes of repairing damaged muscles in the conditions of their natural post-traumatic recovery last for 12–15 d [27].

Anesthesia of rats was carried out by intraperitoneal injection of nembutal (40 mg kg^{-1}), which allowed the animals into deep surgical anesthesia for at least 2 h. *Muscle gastrocnemius* was isolated from surrounding tissues in the area of the hamstring fossa. All branches of the muscle, except for those innervating it, were cut. The isolated muscle was fixed on a bipolar platinum wire electrode for further electrical stimulation. The skin edges on the hind limbs of rats around the incision were sutured to the armature of the strain gauge machine, and the resulting baths with the muscle and nerve were filled with Vaseline oil.

During the experiments, the *muscle gastrocnemius* was transversely dissected by 1, 2, and 3 incisions of 1 mm depth each at three equidistant locations to obtain the 1st, 2nd, and 3rd degree of severity of open muscle injury, respectively [28]. We then sutured the wound skin opening with absorbable synthetic suture material (Liberti, Germany).

2.3. Biomechanical and biochemical studies

Analysis of mechanograms for contractile activity of *muscle gastrocnemius* and biochemical analysis of rat blood were performed 15 d after initiation of open muscle injury.

Stimulation of *muscle gastrocnemius* efferents was carried out by electrical impulses generated using a strain gauge generator. Each series of stimulation consisted of a separate series of rectangular 2 ms pulses with a frequency of 50 Hz and a duration of 6 s. The current strength at which the muscle started to contract was considered as threshold and further stimulation was performed at a strength of 1.3–1.4 threshold. The external load on the muscle was controlled using a mechanostimulator system. The force of muscle contraction was measured using strain gauges [20–23].

In the process of analyzing the obtained results, we used several biomechanical parameters that have a clear tendency to change with increasing severity of muscle pathology [20–23], namely:

- integrated muscle power (S)—numerically calculated area under the power curve using Origin 9.4 software. The analysis of this parameter allows us to evaluate the mechanism of muscle activity formation in the system of equilibrium ‘force of contraction—external load’, which is a physiological analog of the muscular system performance as a whole;
- the level of generation of the maximum force of muscle contraction ($F_{\max}$). This parameter is an indicator of the general dysfunction of the muscular system. A decrease in the maximum possible force response of the muscle is associated with both disorders in the neural component and myotic components of the pathology under study;
- the level of generation of the minimum force of muscle contraction ($F_{\min}$). This parameter is an indicator of the maximum changes caused by the pathological process in each consecutive contractile act;
- an assessment of muscle fatigue development was performed by calculating the time interval until reaching 50% of the force response level (t_{50}). It should be noted that in the control group of animals, very long (several hours) time frames were required to change this parameter.

The concentrations of c-reactive protein (CRP), creatinine, and lactate, as well as indicators of pro- and antioxidant balance (activity of catalase (CAT) and superoxide dismutase (SOD) and concentration of reduced glutathione (GSH)) in the blood plasma and muscle tissue of rats, as markers of muscle damage, were determined using the clinical diagnostic equipment—biochemical analyzers RNL-200 and JN-1101-TR2 (Netherlands), ABX Micros ESV60 and automatic analyzer Pentra C400 (France).

2.4. Statistics

Statistical evaluation of the experimental results was performed using the procedure of analysis of variances with mixed design. Two between-group factors were supposed: (1) open injury (three levels—the 1st, 2nd, and 3rd degree of severity); (2) water-soluble C_{60} fullerenes treatment (two levels—no and use of C_{60}). The factor of consecutive non-relaxation contractions of the *muscle gastrocnemius* when stimulation is applied was supposed as grouped with ten levels. The Shapiro-Wilk W -test was used to test for normality. Levene’s test was used to assess the equality of variances across groups. Multiple pairwise comparisons between different groups and conditions were performed by Bonferroni *post-hoc* test. The differences between the experimental groups were considered significant at $p < 0.05$. Each of the experimental force curves is the result of averaging 10 similar tests. Each biochemical measurement was carried out at least three times. The statistical evaluation was performed by the software package Statistica 8.0 (Dell, USA).

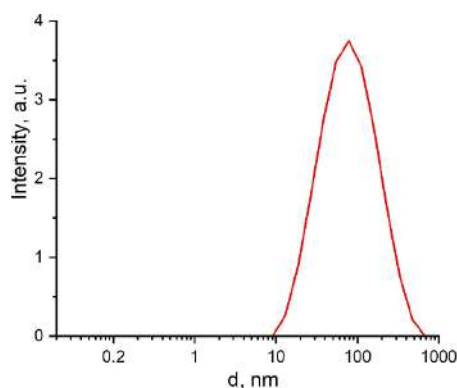


Figure 1. Distribution of the scattered light intensity according to the diameters of light scattering C_{60} fullerene nanoparticles (0.15 mg ml^{-1}).

3. Results and discussion

3.1. AFM analysis

Since the size of nanoparticles affects their toxicity and bioactivity [29], we conducted DLS and AFM research on the resulting sample.

A typical result of DLS experiment shown in figure 1 gives the distribution of light-scattering particles according to their hydrodynamic diameters at a fixed solute concentration (0.15 mg ml^{-1}). The main fraction of light-scattering particles (nanoaggregates of C_{60} fullerenes) had diameters in the range of 100 nm.

The magnitude of the zeta potential is related to the stability of colloid dispersions because it determines the degree and nature of the interaction between the particles of the dispersed system. The zeta potential value for the prepared C_{60} fullerene aqueous solution was equal to -24.7 mV . A high negative charge of colloid clusters (or, more strictly, the electrostatic repulsion between the negatively charged clusters) indicates a low degree of particle aggregation over time.

The AFM image obtained (figure 2(A)) shows large C_{60} fullerene nanoclusters with a size of 20–90 nm, which is in good agreement with above DLS results. Moreover, figure 2(B) demonstrates individual C_{60} molecules (diameter $\sim 0.7 \text{ nm}$) and their small nanoclusters with a size of 1.2–3.0 nm. Thus, the studied aqueous solution is a typical polydisperse nanofluid, which is in good agreement with our previous theoretical calculations [30].

3.2. Biomechanics of injured muscle gastrocnemius contractions

Figure 3 shows the results of changes in the force of 10 consecutive non-relaxation contractions of the *muscle gastrocnemius* of rats 15 d after the initiation of open muscle injury when applying stimulation at a frequency of 50 Hz for a duration of 6 s. The corresponding biomechanical parameters describing the dysfunction of the investigated muscle work were calculated based on the obtained mechanograms (figure 4).

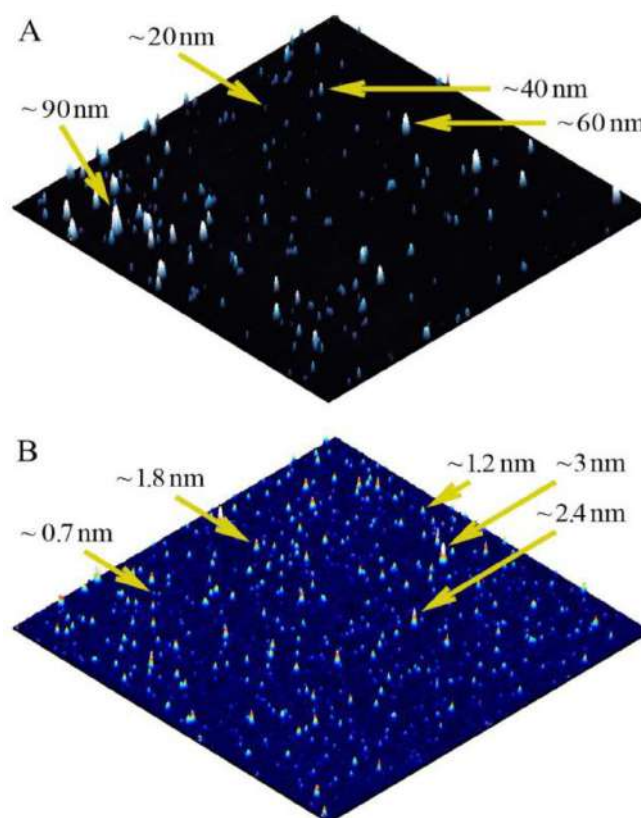


Figure 2. 3D AFM images of C_{60} fullerene nanoparticles in aqueous solution (0.15 mg ml^{-1}).

Change in integrated muscle power is one of the most important parameters of skeletal muscle contraction kinetics [23]. Pathological processes occurring in the nerve or muscle tissue lead to a decrease in this parameter, which indicates the emerging difficulties in accurate joint positioning by the damaged muscle [31].

At the end of 15 d after initiation of open muscle injury of the 1st degree of severity, this parameter was $58 \pm 3\%$ and $38 \pm 2\%$ (control values were taken as 100%) at the first and tenth contraction, respectively. Application of water-soluble C_{60} fullerenes changed the values of these indices, which were $94 \pm 4\%$ and $61 \pm 3\%$, respectively, relative to the control. Significant decrease in integrated muscle power at the 2nd and 3rd degree of injury severity ($44 \pm 2\%$ and $21 \pm 1\%$, and $40 \pm 2\%$ and $15 \pm 1\%$, respectively) was corrected by application of C_{60} fullerenes, namely its increase to $86 \pm 4\%$ and $49 \pm 3\%$ and $50 \pm 3\%$ and $32 \pm 2\%$, respectively, relative to the control (figure 4).

The decrease in integrated muscle power is due to both primary mechanical damage and secondary metabolic damage of myocytes by free radicals. Based on the obtained data, we can assert the positive effect of water-soluble C_{60} fullerenes specifically on secondary, trauma-induced myocyte damage, the recovery of which is usually completed not earlier than after 30 d [32].

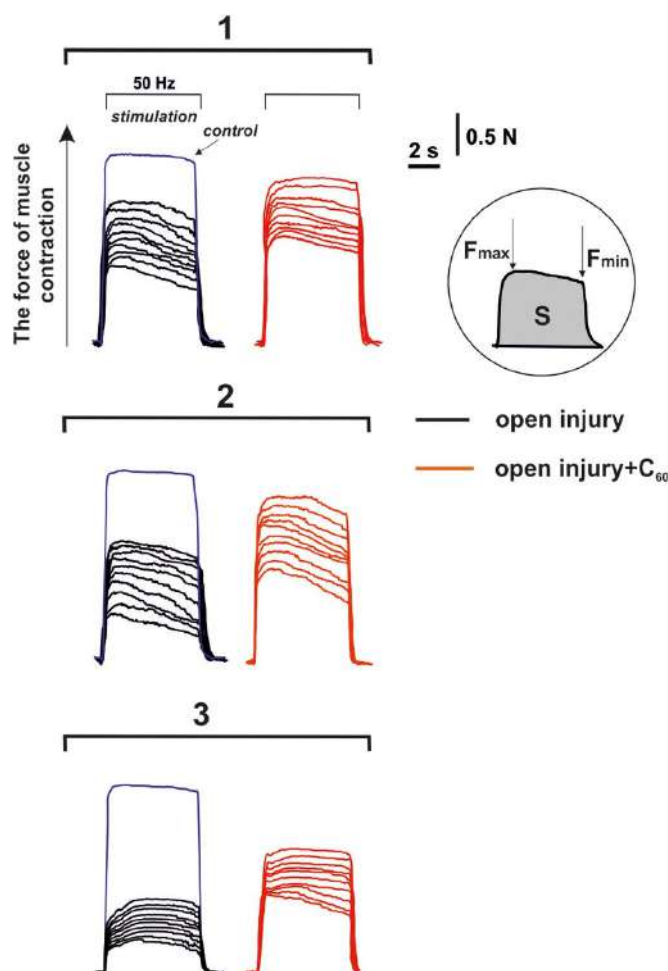


Figure 3. The force of 10 consecutive non-relaxation contractions of rat *muscle gastrocnemius* 15 d after initiation of open muscle injury when applying 50 Hz stimulation of 6 s duration: open injury and open injury + C_{60} —mechanograms of *muscle gastrocnemius* of injured rats and rats receiving water-soluble C_{60} fullerenes after injury initiation; F_{max} and F_{min} —maximum and minimum force of a single muscle contraction, respectively; S —integrated muscle power for a single contraction; 1, 2 and 3—1st, 2nd and 3rd degree of severity of open muscle injury, respectively.

The level of maximum force of muscle contraction is a biomechanical marker responsible for the adequate realization of motoneuron pools by the muscular system. This component of muscle dynamics is particularly important in the control of hand contraction in humans. Increased intramuscular collagen structures, the presence of non-functioning muscle fibers, inflammatory processes, and the involvement of activated neutrophils releasing additional free radicals into the focus of damage reduce the level of this parameter.

15 d after initiation of open muscle injury, this parameter decreased significantly and was 0.68 ± 0.05 and 0.35 ± 0.03 N at the 1st degree of injury severity, 0.54 ± 0.05 and 0.20 ± 0.02 N at the 2nd degree of injury severity, and 0.40 ± 0.03 and 0.18 ± 0.02 N at the 3rd degree of injury severity on the first and tenth contraction, respectively. In the control, its value was 1.2 ± 0.1 N. Application

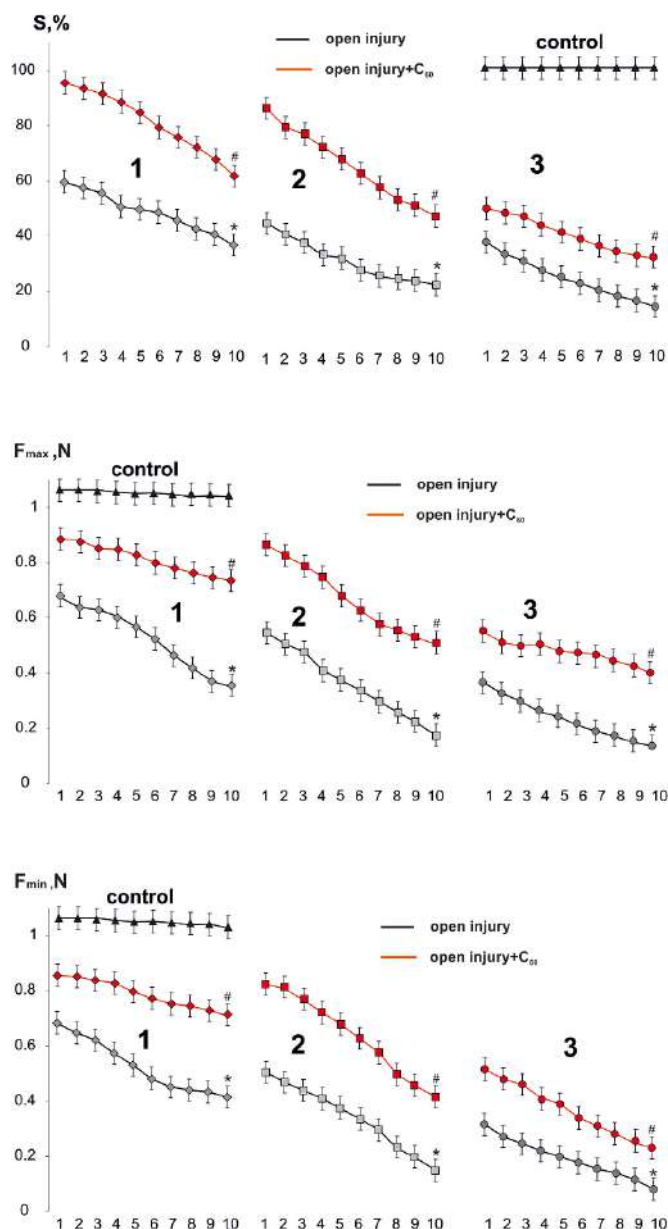


Figure 4. Changes in integrated muscle power, its minimum and maximum force of contraction 15 d after initiation of open injury of rat *muscle gastrocnemius* at the application of 50 Hz stimulation with a duration of 6 s: open injury and open injury + C_{60} —group of injured rats and group of rats receiving water-soluble C_{60} fullerenes after injury initiation, respectively; 1–10—ten consecutive non-relaxation contractions of *muscle gastrocnemius*; 1, 2 and 3—1st, 2nd and 3rd degree of severity of open muscle injury, respectively. * $p < 0.05$ relative to the control group; # $p < 0.05$ relative to the open injury group.

of water-soluble C_{60} fullerenes increased this parameter to 0.89 ± 0.08 and 0.72 ± 0.07 N, 0.90 ± 0.09 and 0.51 ± 0.05 N and 0.55 ± 0.05 and 0.41 ± 0.04 N at the 1st, 2nd and 3rd degree of injury severity, respectively (figure 4).

Note that the results obtained above can be related not only to the protective effect of antioxidants on myocyte membranes [10, 12, 13]. The trajectory of a rather slow muscle movement

is completely determined by its static properties. If the movement is more rapid, dynamic components are added, which directly depend on the stiffness and pliability of the muscle. It is clear that if complications develop after muscle injuries, they undergo significant changes [23]. Increased fibrosis of skeletal muscles during post-traumatic regeneration worsens their function and negatively affects myocyte regeneration after injury, reducing the maximum possible mechanical force [33]. It can be assumed that the use of C_{60} fullerenes can reduce the level of development of such pathological processes.

The level of minimal muscle contraction force is a biomechanical marker describing the quality level of the performed movement. The inability to maintain the achieved target position throughout the entire muscle activation process leads to serious errors in the positioning system and the occurrence of tremor components [34]. This parameter is not associated with neuropathic damage and its analysis gives an idea of the disturbances of the force generation system within the muscle fiber.

After initiation of open muscle injury, the minimum contraction force decreased from a control value of 1.1 ± 0.1 N to 0.69 ± 0.06 and 0.42 ± 0.04 N at the 1st injury severity level, 0.51 ± 0.05 and 0.21 ± 0.02 N at the 2nd injury severity level, and 0.32 ± 0.03 and 0.18 ± 0.01 N at the 3rd injury severity level on the first and tenth contraction, respectively. Application of water-soluble C_{60} fullerenes increased this parameter, which was 0.85 ± 0.08 and 0.73 ± 0.07 N, 0.81 ± 0.08 and 0.42 ± 0.04 N and 0.53 ± 0.05 and 0.30 ± 0.03 N at the 1st, 2nd and 3rd degree of injury severity, respectively (figure 4).

It should be noted that this parameter is significantly affected by the loss of muscle mass that occurs in the process of posttraumatic regeneration. Significant loss of muscle mass by definition exceeds the endogenous ability to regenerate skeletal muscles, which leads to permanent structural and functional deficits [35], and the positive effect of C_{60} fullerenes on atrophic processes in skeletal muscle was confirmed by us earlier [22].

Muscle fatigue is a protective mechanism against overloading the organism and preventing further development of pathological processes in the muscle [36]. Physiological disorders associated with muscle injury increase their fatigue symptomatology and worsen as the severity of injury increases. To evaluate the effect of C_{60} fullerenes on biomechanical parameters of contraction of injured *muscle gastrocnemius* we analyzed their changes at the onset of muscle fatigue.

Figure 5 presents mechanograms of *muscle gastrocnemius* contraction in rats 15 d after initiation of open muscle injury when applying stimulation at a frequency of 50 Hz for a duration of 2 s in the case of 100 consecutive non-relaxation contractions. On their basis, the indices of the integrated muscle power and reduction time of the maximal force response to 50% of the initial amplitude were analyzed (figure 6).

The integrated muscle power throughout the entire period of stimulation decreased to $45 \pm 3\%$, $40 \pm 2\%$, and $30 \pm 2\%$ at the 1st, 2nd, and 3rd degree of injury severity, respectively, relative to the control. It should be noted that at the 3rd degree

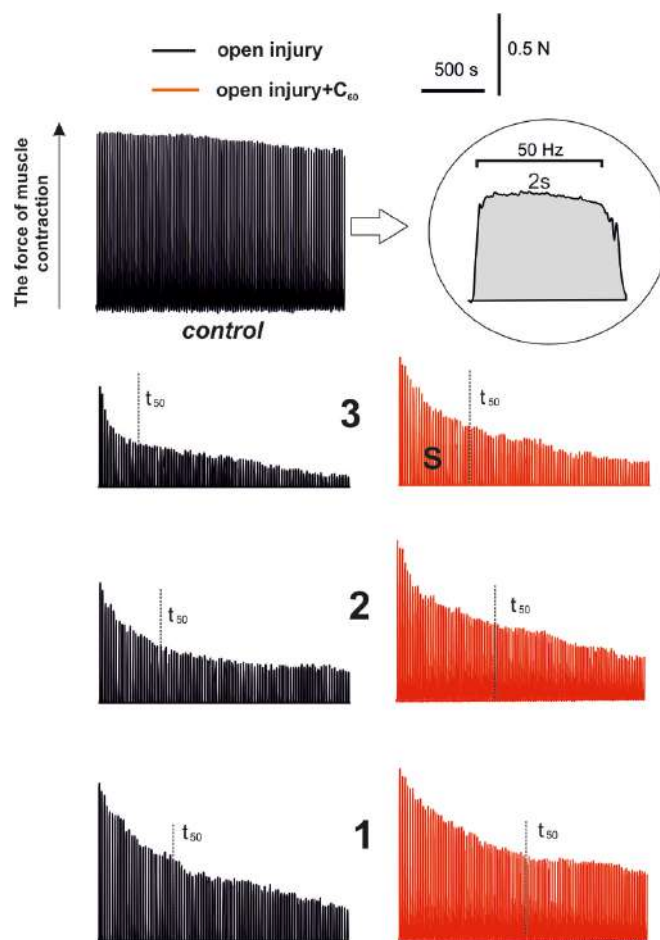


Figure 5. Force generation curves of *muscle gastrocnemius* contractions in rats 15 d after initiation of open muscle injury when applying stimulation at a frequency of 50 Hz for a duration of 2 s in the case of 100 consecutive non-relaxation contractions: open injury and open injury + C_{60} —mechanograms of *muscle gastrocnemius* of injured rats and rats receiving water-soluble C_{60} fullerenes after injury initiation; 1, 2 and 3—1st, 2nd and 3rd degree of severity of open muscle injury, respectively; S—integrated muscle power for the whole contractile process; t_{50} —time to reduce the maximal force response to 50% of the initial muscle force amplitude.

of injury severity the reduction of this parameter after 1200 s ended with almost complete muscle stiffness. Application of water-soluble C_{60} fullerenes increased this parameter, which was $74 \pm 4\%$, $58 \pm 3\%$, and $47 \pm 2\%$ at the 1st, 2nd, and 3rd degree of injury severity, respectively, relative to the control. Note that in this case, the muscle responded with a contractile response throughout the entire period of stimulation, not decreasing below the $30 \pm 2\%$ limit (figure 5).

The time to decrease the force response of the muscle by 50% of the initial value was 609 ± 11 , 501 ± 13 , and 408 ± 8 s at the 1st, 2nd, and 3rd degree of injury severity, respectively. Application of water-soluble C_{60} fullerenes significantly increased this parameter, which was 1011 ± 12 , 759 ± 15 , and 575 ± 9 s at the 1st, 2nd, and 3rd degree of injury severity, respectively (figure 6).

In our opinion, the improvement of these biomechanical parameters of contraction of injured *muscle gastrocnemius* of

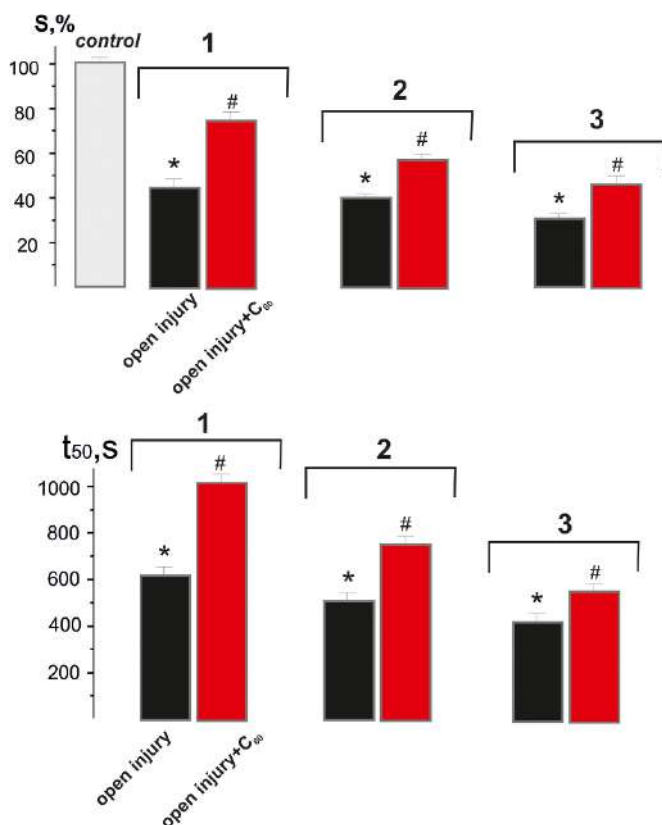


Figure 6. Changes in integrated muscle power and time of maximum force response reduction to 50% of the initial amplitude 15 d after initiation of open injury of rat *muscle gastrocnemius* at the application of stimulation with a frequency of 50 Hz with duration of 2 s in case of 100 consecutive non-relaxation contractions: open injury and open injury + C₆₀—group of injured rats and group of rats receiving water-soluble C₆₀ fullerenes after injury initiation, respectively; 1, 2 and 3—1st, 2nd and 3rd degree of severity of open muscle injury, respectively. * $p < 0.05$ relative to the control group; # $p < 0.05$ relative to the open injury group.

rats upon application of C₆₀ fullerenes can be explained precisely by their antioxidant properties [37].

In summary, the application of water-soluble C₆₀ fullerenes in a daily dose of 1 mg kg⁻¹ during the whole experiment increases the values of investigated mechanokinetic parameters of contraction of injured *muscle gastrocnemius* of rats at the level of $30\text{--}45 \pm 3\%$ relative to the open injury group.

3.3. Blood and muscle tissue biochemical indicators of rats

To confirm the obtained mechanokinetic data on the contraction of injured *muscle gastrocnemius* at the application of C₆₀ fullerenes we analyzed the changes of blood and muscle tissue biochemical composition of experimental animals.

Highly sensitive CRP is a clinical marker of both acute and chronic phases of inflammation in musculoskeletal diseases [38]. It is known that CRP concentration increases in response to the development of muscle injury.

The recorded significant increase in CRP concentration from 0.50 ± 0.03 mg l⁻¹ in control to 1.8 ± 0.1 , 2.7 ± 0.2 ,

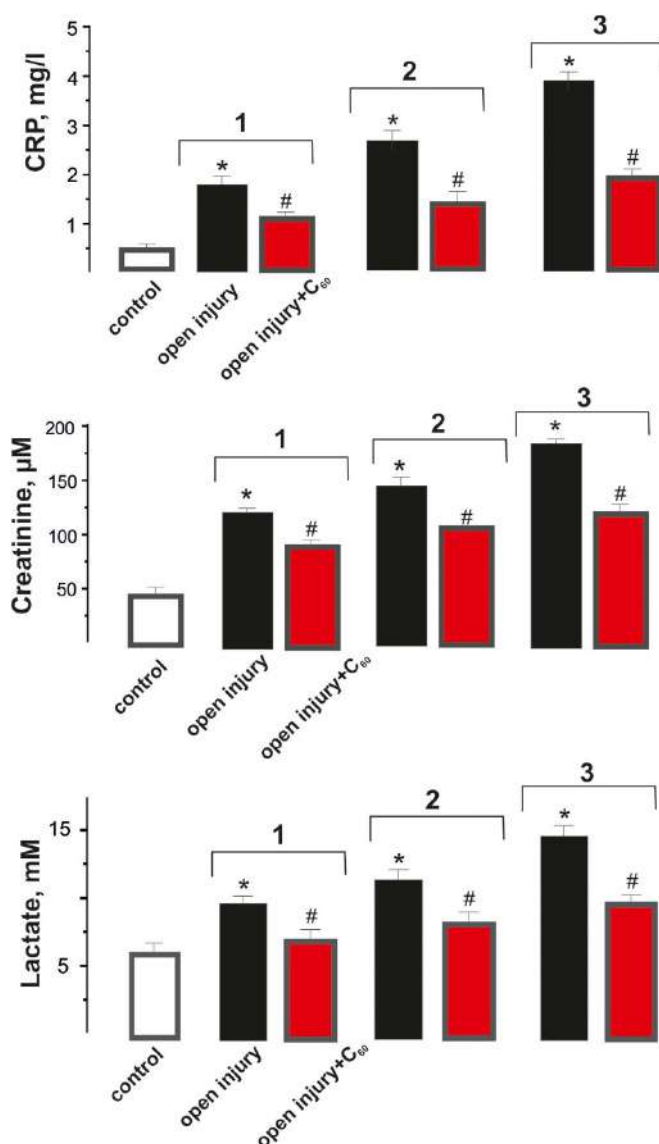


Figure 7. CRP, creatinine, and lactate concentrations in rat blood plasma 15 d after initiation of open *muscle gastrocnemius* injury: open injury and open injury + C₆₀—a group of injured rats and a group of rats receiving water-soluble C₆₀ fullerenes after injury initiation, respectively; 1, 2 and 3—1st, 2nd and 3rd degree of severity of open muscle injury, respectively. * $p < 0.05$ relative to the control group; # $p < 0.05$ relative to the open injury group.

and 3.9 ± 0.3 mg l⁻¹ at the 1st, 2nd, and 3rd degree of injury severity, respectively, is evidence of ongoing inflammatory processes in the injured muscle. Application of water-soluble C₆₀ fullerenes significantly decreased its concentration to 1.1 ± 0.1 , 1.4 ± 0.1 , and 2.0 ± 0.2 mg l⁻¹ at the 1st, 2nd and 3rd degree of injury severity, respectively (figure 7), which is a confirmation of the prospective application of antioxidants in the therapy of muscle pathologies [17–20].

Creatinine, as a source of energy for muscle contraction, is the end product of creatine and creatine phosphate metabolism. Changes in its concentration are caused by myocyte destruction under various physiological and pathological conditions [39].

Creatinine concentration increased from $43 \pm 2 \mu\text{M}$ in control to 122 ± 6 , 149 ± 5 , and $181 \pm 7 \mu\text{M}$ at the 1st, 2nd, and 3rd degree of injury severity, respectively. Application of water-soluble C_{60} fullerenes significantly reduced this index to 82 ± 2 , 104 ± 3 , and $119 \pm 3 \mu\text{M}$, respectively (figure 7), which, in our opinion, is due to antioxidant properties of C_{60} fullerenes, which protect skeletal muscle cell membranes from nonspecific free-radical destruction by actively absorbing free radicals.

Skeletal muscles are the main producer of lactic acid in the body, but their fibers also use lactic acid as a breathing fuel. The transport of lactic acid across the plasma membrane is fundamental to intracellular pH homeostasis. Lactate is an important metabolic intermediate that can be rapidly exchanged between different cells within a given muscle, between different muscles, and between muscle and blood [40]. An increase in lactate concentration indicates that the amount of lactate entering the cell exceeds its oxidation and excretion. This may be a direct consequence of muscle fiber dysfunction associated with impaired reparative processes after injury.

In control, the lactate concentration was $5.3 \pm 0.5 \text{ mM}$. After initiation of open muscle injury, its value increased to 9.2 ± 0.7 , 11.9 ± 0.7 , and $14.3 \pm 0.8 \text{ mM}$ at the 1st, 2nd, and 3rd degree of injury severity, respectively. Application of water-soluble C_{60} fullerenes contributed to the increase of lactate oxidation: its concentration significantly decreased and was 7.1 ± 0.3 , 7.7 ± 0.3 , and $9.8 \pm 0.4 \text{ mM}$ at the 1st, 2nd and 3rd degree of injury severity, respectively (figure 7).

Muscle damage is usually accompanied by the formation of free oxygen radicals and the intensification of lipid peroxidation (LPO) processes [41]. As a result of biochemical tests, we recorded an increase in the level of markers of peroxidation and oxidative stress (SOD, CAT, and GSH) in the blood and muscle tissue of rats after the induction of injury and their decrease upon application of C_{60} fullerenes (figures 8 and 9).

SOD is one of the most potent endogenous antioxidants, which protects body cells from the damaging effects of free radicals formed during the activation of LPO [42].

SOD activity in blood was 3.7 ± 0.2 , 4.3 ± 0.4 , and $5.8 \pm 0.4 \text{ Units ml}^{-1}$ at the 1st, 2nd, and 3rd degree of injury severity, respectively, and $1.8 \pm 0.1 \text{ Units ml}^{-1}$ in the control. Application of water-soluble C_{60} fullerenes significantly reduced this rate, which was 2.4 ± 0.1 , 2.8 ± 0.2 , and $3.2 \pm 0.3 \text{ Units ml}^{-1}$ at the 1st, 2nd, and 3rd degree of injury severity, respectively (figure 8).

SOD activity in muscle tissue was 4.3 ± 0.3 , 5.7 ± 0.6 , and $8.2 \pm 0.5 \text{ Units mg}^{-1} \text{ protein}$ at the 1st, 2nd, and 3rd degree of injury severity, respectively, and $2.6 \pm 0.2 \text{ Units mg}^{-1} \text{ protein}$ in the control. Application of water-soluble C_{60} fullerenes significantly reduced this rate, which was 3.1 ± 0.2 , 4.0 ± 0.3 , and $5.2 \pm 0.4 \text{ Units mg}^{-1} \text{ protein}$ at the 1st, 2nd, and 3rd degree of injury severity, respectively (figure 9).

CAT is an enzyme that protects body cells from oxidative damage by reactive oxygen species. The significance of CAT can be judged by assessing its direct or indirect involvement in various diseases [43].

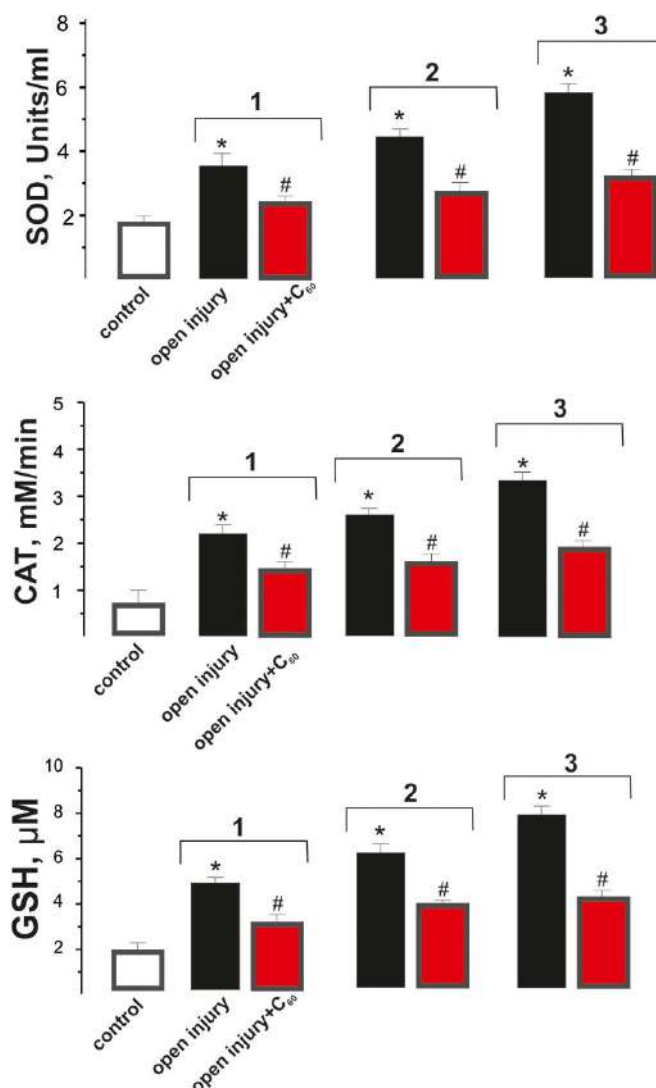


Figure 8. Indices of pro- and antioxidant balance (SOD, CAT, and GSH) in rat blood plasma 15 d after initiation of open injury of *muscle gastrocnemius*: open injury and open injury + C_{60} —a group of injured rats and a group of rats receiving water-soluble C_{60} fullerenes after injury initiation, respectively; 1, 2 and 3—the 1st, 2nd and 3rd degree of severity of open muscle injury, respectively. * $p < 0.05$ relative to the control group; # $p < 0.05$ relative to the open injury group.

CAT activity in blood after initiation of open muscle injury increased from $0.60 \pm 0.05 \text{ mM min}^{-1}$ in control to 2.2 ± 0.1 , 2.5 ± 0.2 , and $3.4 \pm 0.3 \text{ mM min}^{-1}$ at the 1st, 2nd, and 3rd degree of injury severity, respectively. Application of water-soluble C_{60} fullerenes reduced this index to 1.4 ± 0.1 , 1.6 ± 0.1 and $1.9 \pm 0.2 \text{ mM min}^{-1}$, respectively (figure 8).

CAT activity in muscle tissue increased from $2.0 \pm 0.3 \mu\text{M/min/mg protein}$ in control to 3.5 ± 0.3 , 4.4 ± 0.4 , and $5.2 \pm 0.5 \mu\text{M/min/mg protein}$ at the 1st, 2nd, and 3rd degree of injury severity, respectively. Application of water-soluble C_{60} fullerenes reduced this index to 2.8 ± 0.2 , 3.2 ± 0.3 and $3.9 \pm 0.4 \mu\text{M/min/mg protein}$, respectively (figure 9).

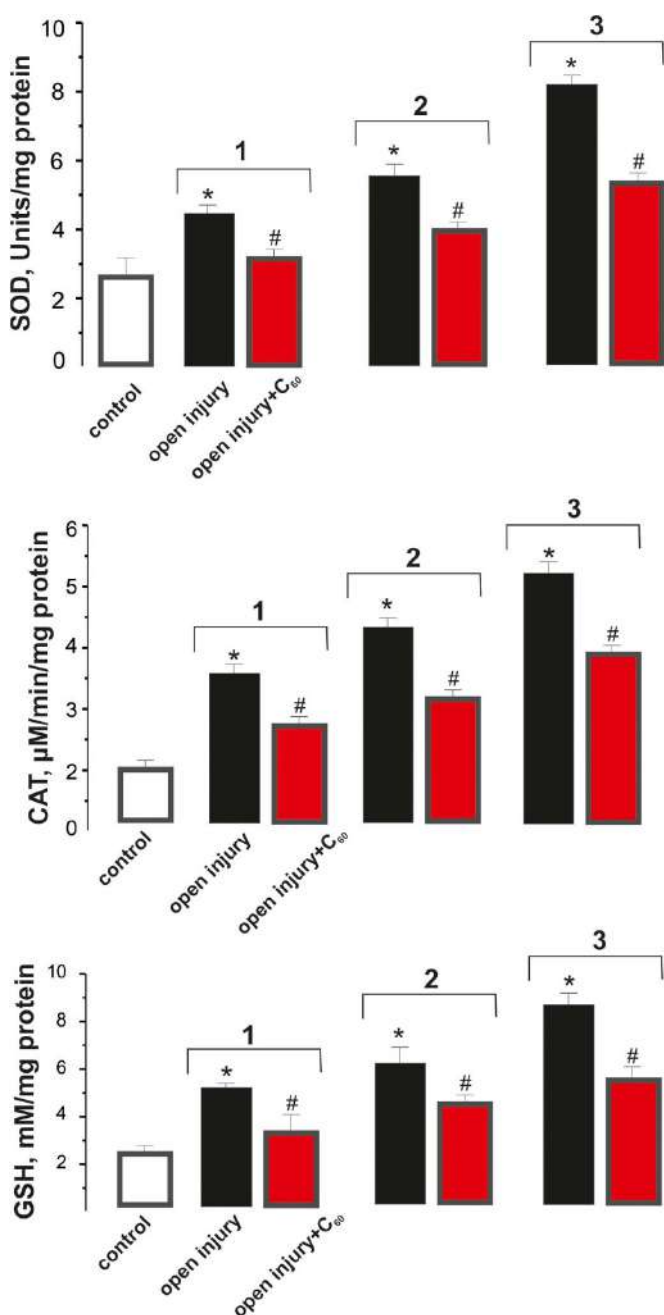


Figure 9. Indices of pro- and antioxidant balance (SOD, CAT, and GSH) in rat *muscle gastrocnemius* tissue 15 d after initiation of open injury: open injury and open injury + C₆₀—a group of injured rats and a group of rats receiving water-soluble C₆₀ fullerenes after injury initiation, respectively; 1, 2 and 3—the 1st, 2nd and 3rd degree of severity of open muscle injury, respectively. * $p < 0.05$ relative to the control group; # $p < 0.05$ relative to the open injury group.

When free radicals are excessively produced, GSH reserves are sharply depleted. The presence of sufficient GSH concentration is a critical factor for cell survival under oxidative stress [44].

After initiation of open muscle injury, GSH concentration in blood was 4.9 ± 0.4 , 6.1 ± 0.4 , and 7.9 ± 0.5 μM at the

1st, 2nd, and 3rd degree of injury severity, respectively, and 1.9 ± 0.1 μM in the control. Application of water-soluble C₆₀ fullerenes reduced this index to 3.1 ± 0.2 , 3.9 ± 0.3 and 4.2 ± 0.3 μM , respectively (figure 8).

GSH concentration in muscle tissue was 5.1 ± 0.5 , 6.2 ± 0.6 , and 8.9 ± 0.8 mM mg^{-1} protein at the 1st, 2nd, and 3rd degree of injury severity, respectively, and 2.2 ± 0.4 $\mu\text{M mg}^{-1}$ protein in the control. Application of water-soluble C₆₀ fullerenes reduced this index to 3.2 ± 0.3 , 4.7 ± 0.4 and 5.8 ± 0.6 mM mg^{-1} protein, respectively (figure 9).

Thus, the application of water-soluble C₆₀ fullerenes in a daily dose of 1 mg kg^{-1} during the whole experiment reduces biochemical indices in the blood and muscle tissue of experimental animals at the level of $35\text{--}50 \pm 3\%$ and $20\text{--}37 \pm 3\%$, correspondingly, relative to the open injury group, thus contributing to the reduction of oxidative processes in the damaged muscles by maintaining the balance between pro-oxidants and antioxidant defense system, preventing the negative effect of free radicals on cellular and subcellular structures during reparative post-traumatic processes in rats.

4. Conclusions

Based on the obtained data, we can conclude that application of water-soluble C₆₀ fullerenes (daily oral dose 1 mg kg^{-1}) within 15 d after initiation of open injury of *muscle gastrocnemius* of rats of different severity degree improves its contractile activity, which is confirmed by the increase of values of the studied biomechanical parameters of muscle contractions at the level of $30\text{--}45 \pm 3\%$ and decrease of biochemical indices in the blood and muscle tissue at the level of $35\text{--}50 \pm 3\%$ and $20\text{--}37 \pm 3\%$, correspondingly, relative to the open injury group. In our opinion, C₆₀ fullerenes can influence the activity of endogenous antioxidants, preventing the occurrence of dysfunction in the active muscle and, thus, maintaining it within the physiological norm during the whole process of its contraction. This opens up the possibility of using C₆₀ fullerenes as promising nanoparticles—oxidants capable of correcting pathological conditions of the muscular system arising during the repair of open injuries.

Data availability statement

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgment

This research was supported by the National Research Foundation of Ukraine (2022.01/0004).

Author contributions

Conceptualization, Y Prylutsky; Formal analysis, O Motuziuk, and D Nozdrenko; Investigation, D Nozdrenko, O Motuziuk, S Prylutska, N Nurishchenko, D Franskevych, V SoroCa, V Cherepanov, I Kalinin, O Korzhyk; Coordination of the research work, Y Prylutsky and D Nozdrenko; Writing—original draft preparation, D Nozdrenko; Writing—review and editing, Y Prylutsky and U Ritter; Supervision, U Ritter.

Conflict of interest

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

All of the *in vivo* experiments were approved by the Bioethics Committee of the ESC ‘Institute of Biology and Medicine’ of Taras Shevchenko National University of Kyiv (protocol No. 9 dated 4 September 2023) and performed under the ‘European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes’ (Strasbourg, 1986) and Article 26 of the Law of Ukraine ‘On the Protection of Animals from Cruelty’ (No. 3447-IV, 21.02.2006), as well as European Union Directive of 22 September 2010 (2010/63/EU) for the protection of animals used for scientific purposes.

ORCID iDs

Yuriy Prylutsky  <https://orcid.org/0000-0002-9847-4137>

Dmytro Nozdrenko  <https://orcid.org/0000-0001-7254-1533>

Olexandr Motuziuk  <https://orcid.org/0000-0002-1520-0721>

Svitlana Prylutska  <https://orcid.org/0000-0001-5280-8341>

Natalia Nurishchenko  <https://orcid.org/0000-0001-8160-8832>

Daria Franskevych  <https://orcid.org/0000-0002-1719-0512>

Vasil SoroCa  <https://orcid.org/0000-0002-8340-7636>

Vsevolod Cherepanov  <https://orcid.org/0000-0003-4649-1936>

Ihor Kalinin  <https://orcid.org/0000-0002-3740-7600>

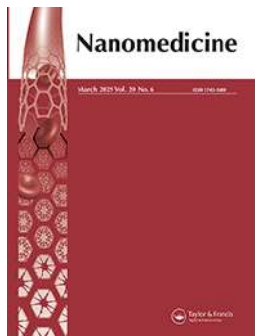
Olha Korzhyk  <https://orcid.org/0000-0002-2526-8877>

Uwe Ritter  <https://orcid.org/0000-0002-9315-4863>

References

- [1] Järvinen T A H, Järvinen T L N, Kääriäinen M, Aärimaa V, Vaittinen S, Kalimo H and Järvinen M 2007 *Best Pract. Res.: Clin. Rheumatol.* **21** 317
- [2] Gottipati S, Gowtham B, Chalimeda S, Ravipati S, Vishaal P and Khullar K 2024 *Cureus* **16** e54468
- [3] Bartlett C S, Helfet D L, Hausman M R and Strauss E 2000 *J. Am. Acad. Orthop. Surg.* **8** 21
- [4] Dougherty P J, Najibi S, Silverton C and Vaidya R 2009 *Instr. Course Lect.* **58** 131
- [5] Bulla A, Delgove A, De Luca L, Pelissier P and Casoli V 2020 *Ann. Chir. Plast. Esthet.* **65** 655
- [6] Myburgh K H, Kruger M J and Smith C 2012 *J. Nutr. Biochem.* **23** 1072
- [7] Hurtgen B J, Ward C L, Garg K, Pollot B E, Goldman S M, McKinley T O, Wenke J C and Corona B T 2016 *J. Musculoskelet. Neuronal Interact.* **16** 122
- [8] Smith C, Kruger M J, Smith R M and Myburgh K H 2008 *Sports Med.* **38** 947
- [9] Webster J, Scuffham P, Sherriff K L, Stankiewicz M and Chaboyer W P 2012 *Cochrane Database Syst. Rev.* **4** CD009261
- [10] Kozakowska M, Pietraszek-Gremplewicz K, Jozkowicz A and Dulak J 2015 *J. Muscle Res. Cell Motil.* **36** 377
- [11] Madrigal P, Moshal T, Bernabe R, Yenikomshian H and Gillenwater J 2022 *J. Tissue Viability* **31** 630
- [12] Ohmae S, Akazawa S, Takahashi T, Izumo T, Rogi T and Nakai M 2022 *Biochem. Biophys. Res. Commun.* **615** 24
- [13] Hsu Y-J, Ho C-S, Lee M-C, Ho C-S, Huang C-C and Kan N-W 2020 *J. Med. Sci.* **17** 53
- [14] Lee J E, Shah V K, Lee E-J, Oh M-S and Choi J J 2019 *J. Pharmacol. Sci.* **140** 26
- [15] Gharbi M, Pressac M, Hadchouel M, Szwarc H, Wilson S R and Moussa F 2005 *Nano Lett.* **5** 2578
- [16] Prylutska S V, Grynuk I I, Matyshevska O P, Prylutsky Y I, Ritter U and Scharff P 2008 *Fullerenes Nanotubes Carbon Nanostruct.* **16** 698
- [17] Kuznietsova H, Dziubenko N, Hurmach V, Chereschuk I, Motuziuk O, Ogloblya O and Prylutsky Y 2020 *Oxid. Med. Cell. Long.* **2020** 8061246
- [18] Eswaran S V 2018 *Curr. Sci.* **114** 1846
- [19] Vereshchaka I V et al 2018 *Front. Physiol.* **9** 517
- [20] Nozdrenko D, Matvienko T, Vygovska O, Bogutska K, Motuziuk O, Nurishchenko N, Prylutsky Y, Scharff P and Ritter U 2021 *Int. J. Mol. Sci.* **22** 6812
- [21] Nozdrenko D, Abramchuk O, Prylutska S, Vygovska O, SoroCa V, Bogutska K, Khrapatyi S, Prylutsky Y, Scharff P and Ritter U 2021 *Int. J. Mol. Sci.* **22** 4977
- [22] Nozdrenko D, Prylutska S, Bogutska K, Nurishchenko N, Abramchuk O, Motuziuk O, Prylutsky Y, Scharff P and Ritter U 2022 *Life* **12** 332
- [23] Nozdrenko D, Motuziuk O, Prylutska S, Matvienko T, Bogutska K, Franskevych D, Nurishchenko N, Abramchuk O and Prylutsky Y 2024 *Heliyon* **10** e32677
- [24] Prylutska S V, Matyshevska O P, Grynuk I I, Prylutsky Y, Ritter U and Scharff P 2007 *Mol. Cryst. Liq. Cryst.* **468** 265
- [25] Ji Z Q, Sun H, Wang H, Xie Q, Liu Y and Wang Z 2006 *J. Nanopart. Res.* **8** 53
- [26] Nikolic N, Vranjes-Ethurić S, Janković D, Ethokic D, Mirković M, Bibić N and Trajković V 2009 *Nanotechnology* **20** 385102
- [27] Best T M and Hunter K D 2000 *Phys. Med. Rehabil. Clin.* **11** 251
- [28] Sicherer S T, Venkatarama R S and Grasman J M 2020 *Bioengineering* **7** 76

- [29] Abbasi R, Shineh G, Mobaraki M, Doughty S and Tayebi L 2023 *J. Nanopart. Res.* **25** 43
- [30] Prilutski Y I, Durov S S, Yashchuk V N, Ogul'chansky T Y, Pogorelov V E, Astashkin Y A, Buzaneva E V, Kirghizov Y, Andrievsky G V and Scharff P 1999 *Eur. J. Phys.* **9** 341
- [31] Fitts R H, McDonald K S and Schluter J M 1991 *J. Biomech.* **24** 111
- [32] Faulkner J A, Brooks S V and Opiteck J A 1993 *Phys. Ther. Rehabil. J.* **73** 911
- [33] Mahdy M A A 2019 *Cell Tissue Res.* **375** 575
- [34] Moyer A L and Wagner K R 2011 *Curr. Opin. Rheumatol.* **23** 568
- [35] Mintz E L, Passipieri J A, Franklin I R, Toscano V M, Afferton E C, Sharma P R and Christ G J 2020 *Tissue Eng. A* **26** 140
- [36] Johansson H, Windhorst U, Djupsjöbacka M and Passatore M 2023 (Gävle University Press) 291
- [37] Prylutsky Y, Nozdrenko D, Gonchar O, Prylutska S, Bogutska K, Täuscher E, Scharff P and Ritter U 2023 *J. Nanomater.* **22** 37574
- [38] Macphail K 2015 *Int. Musculoskelet. Med.* **37** 29
- [39] Baxmann A C, Ahmed M S, Marques N C, Menon V B, Pereira A B, Kirsztajn G M and Heilberg I P 2008 *Clin. J. Am. Soc. Nephrol.* **3** 348
- [40] Juel C and Halestrap A P 1999 *J. Physiol.* **517** 633
- [41] Cuzzocrea S, Riley D P, Caputi A P and Salvemini D 2001 *Pharmacol. Rev.* **53** 135
- [42] Islam M N, Rauf A, Fahad F I, Emran T B, Mitra S, Olatunde A, Shariati M A, Rebezov M, Rengasamy K R R and Mubarak M S 2022 *Crit. Rev. Food Sci. Nutr.* **62** 7282
- [43] Lismont C, Revenco I and Fransen M 2019 *Int. J. Mol. Sci.* **20** 3673
- [44] Weschawalit S, Thongthip S, Phutrakool P and Asawanonda P 2017 *Clin. Cosmet. Invest. Dermatol.* **10** 147



C₆₀ fullerene promotes post-traumatic recovery of the rat *muscle gastrocnemius*

Yuriy Prylutsky, Dmytro Nozdrenko, Olexandr Motuziuk, Svitlana Prylutska, Igor Vareniuk, Natalia Nurishchenko, Daria Franskevych, Vasil Soroca, Kateryna Bogutska & Uwe Ritter

To cite this article: Yuriy Prylutsky, Dmytro Nozdrenko, Olexandr Motuziuk, Svitlana Prylutska, Igor Vareniuk, Natalia Nurishchenko, Daria Franskevych, Vasil Soroca, Kateryna Bogutska & Uwe Ritter (2025) C₆₀ fullerene promotes post-traumatic recovery of the rat *muscle gastrocnemius*, Nanomedicine, 20:6, 571-584, DOI: [10.1080/17435889.2025.2461988](https://doi.org/10.1080/17435889.2025.2461988)

To link to this article: <https://doi.org/10.1080/17435889.2025.2461988>



© 2025 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.



[View supplementary material](#)



Published online: 11 Feb 2025.



[Submit your article to this journal](#)



Article views: 742



[View related articles](#)



[View Crossmark data](#)



Citing articles: 1 [View citing articles](#)

C₆₀ fullerene promotes post-traumatic recovery of the rat *muscle gastrocnemius*

Yuriy Prylutskyi ^a, Dmytro Nozdrenko ^a, Olexandr Motuziuk ^{a,b}, Svitlana Prylutska ^c, Igor Varenjuk ^a,
Natalia Nurishchenko ^a, Daria Franskevych ^a, Vasil Soroca ^a, Kateryna Bogutska ^a and Uwe Ritter ^d

^aESC “Institute of Biology and Medicine”, Taras Shevchenko National University of Kyiv, Kyiv, Ukraine; ^bFaculty of Biology and Forestry, Lesya Ukrainka Volyn National University, Lutsk, Ukraine; ^cFaculty of Plant Protection, Biotechnology and Ecology, National University of Life and Environmental Science of Ukraine, Kyiv, Ukraine; ^dInstitute of Chemistry and Biotechnology, Technical University of Ilmenau, Ilmenau, Germany

ABSTRACT

Aim: The remarkable antioxidant capabilities of biocompatible and safe C₆₀ fullerenes have extensive applications in biomedicine. This study is the first to present an investigation into the effect of water-soluble C₆₀ fullerenes on post-traumatic recovery of the *muscle gastrocnemius* in rats.

Methods: Tensometry was used to investigate the biomechanical parameters of muscle contraction, specifically the times of reaching and holding the maximum force response of the muscle, and the return of muscle contraction force to the initial value after cessation of stimulation. Blood biochemical indicators were assessed, including concentrations of c-reactive protein, lactate, creatinine, and reduced glutathione, as well as superoxide dismutase and catalase activities 5, 10, and 15 days after initiating open muscle injury. Histopathological analysis was also performed to examine the rat *muscle gastrocnemius* damage on day 15 after the onset of injury.

Results: It was found that C₆₀ fullerenes reduced the stiffness of injured skeletal muscle, thereby slowing the development of fibrosis, and inhibiting the inflammatory process due to their antioxidant properties. There was also a reduction in histopathological features of muscle damage.

Conclusion: These findings suggest using of carbon nanoparticles to correct pathological conditions that may occur during the physiological repair of damaged muscle tissues.

ARTICLE HISTORY

Received 15 August 2024

Accepted 30 January 2025

KEYWORDS

C₆₀ fullerene; *muscle gastrocnemius*; open muscle injury; biomechanical parameters of muscle contraction; biochemical indicators of blood; histopathological analysis

1. Background

Open injuries are the most frequent and severe type of skeletal muscle damage [1,2]. Inadequate treatment of such injuries, especially at an early stage, contributes to the development of subsequent pathologies of the injured muscle and even loss of its performance [3,4]. Today, there is no unvarying approach to classifying muscle injuries [5], and there is limited evidence to correlate any of their clinical features with the extent of pathology found [6]. This problem is further complicated by the fact that patients with open muscle injuries sustained in extreme conditions often present with a combination of classic major trauma, including both blunt and penetrating wounds, resulting in an increased level of inflammation [7]. Therefore, a comprehensive management strategy for open muscle injuries should include “mitigation” of their consequences at a systemic level as well as direct restoration of the resulting musculoskeletal dysfunction [8,9]. Moreover, novel methods such as dermal substitutes, external tissue expansion, and regenerative matrices used in the treatment of injured muscles can lead to increased fibrotic formation and consequently increased muscle stiffness [10].

The process of repairing damaged muscle tissue involves the coordinated activity of different cell types in response to local and

systemic signals. This process requires the participation of several populations of resident muscle cells, including satellite stem cells, fibroblasts, macrophages, and vascular cells. Infiltrating inflammatory cells, together with resident stem cells, play a critical role in restoring homeostasis to the injured muscle. Abnormal repair mechanisms in dystrophic muscle and the final stage of this process, fibrosis, is a common endpoint for almost all open muscle injuries [11]. At the same time, high-severity injuries cause the simultaneous destruction of many tissue components, surpassing the intrinsic capacity for wound healing without scarring and leading to permanent functional deficits. In this case, the nonproductive inflammatory response and associated fibrosis dominate [12]. This problem is further complicated by the fact that, as part of the inflammatory process, stem cell capacity is significantly inhibited and fibrogenic cells are continuously activated. This ultimately leads to the conversion of muscle tissue into nonfunctional fibrotic tissue. Increased fibrosis of skeletal muscle impairs its function and negatively affects regeneration after injury. Consequently, the formation of fibrosis is considered to be a major contributor to muscle weakness [13]. If muscle injury is not adequately treated, fibrosis can become a self-sustaining process that prevents complete muscle regeneration [14]. In addition, if tissue damage persists over a prolonged period – characteristic of the inflammatory

CONTACT Uwe Ritter  uwe.ritter@tu-ilmenau.de  Institute of Chemistry and Biotechnology, Technical University of Ilmenau, Weimarer Str. 25, Ilmenau 98693, Germany

 Supplemental data for this article can be accessed online at <https://doi.org/10.1080/17435889.2025.2461988>

© 2025 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (<http://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way. The terms on which this article has been published allow the posting of the Accepted Manuscript in a repository by the author(s) or with their consent.

Article highlights

- A C₆₀ fullerene aqueous solution (C₆₀FAS) contains mainly individual C₆₀ molecules and their nanoaggregates
- C₆₀FAS improves biomechanical parameters of rat *muscle gastrocnemius* contraction after open injury
- C₆₀FAS attenuates fibrosis in rat *muscle gastrocnemius* after open injury
- C₆₀FAS improves rat blood biochemical indicators after open muscle injury
- C₆₀FAS reduces in histopathological features of rat *muscle gastrocnemius* damages

process associated with severe open muscle injury – the muscle regeneration process becomes dysregulated. This leads to the replacement of myofibrils with a nonfunctional mass of fibrous tissue [15].

Antioxidant therapy is used quite effectively to reduce the inflammatory processes in muscle injury [16]. So, resveratrol exhibits a protective effect on contusion induced muscle injury and improves its regeneration compared to conventional treatment with non-steroidal anti-inflammatory drugs [17]. Melittin enhances muscle regeneration factors expression in a mouse model of skeletal muscle contusion [18]. The authors [19] found that a hydroxyl radical scavenger, EPC-K1, reduces reperfusion injury in rat skeletal muscle. Finally, quercetin attenuates adipogenesis and fibrosis in human skeletal muscle [20].

It has been shown that water-soluble and nontoxic (at least at low doses) C₆₀ fullerenes effectively protect cell membranes from oxidation by capturing free radicals before their interaction with lipids [21,22]. On *in vivo* models, it was found that C₆₀ fullerene has more potent antioxidant properties compared to such well-known natural antioxidants as vitamins C, E, and carotenoids [23], as well as the exogenous antioxidant N-acetylcysteine [24], which often uses in sports medicine. As a result, its use in modeling ischemia-reperfusion, closed injury, and atrophy of the rat *muscle soleus* leads to significant positive therapeutic effects [25–27].

Thus, the present work aimed to evaluate the water-soluble C₆₀ fullerenes impact on the post-traumatic recovery of rat *muscle gastrocnemius* by analyzing the selective biomechanical parameters of its contraction and pathohistological features of damages, as well as the biochemical indicators of animal blood after open muscle injury initiation.

2. Materials & methods

2.1. Preparation of nanofluid & its characterization

C₆₀ fullerene (Sigma-Aldrich, purity > 99.95%) aqueous solution (C₆₀FAS) has been prepared according to the ultrasonic method described in detail in [28,29]. The resulting C₆₀FAS (maximum concentration was 0.15 mg/ml) is stable for 12–18 months at a temperature of +4–25°C [30].

Structural study of water-soluble C₆₀ fullerene particles was performed by atomic force microscopy (AFM; NT-MDT, Apeldoorn, Netherlands) [30]. A drop of sample was

transferred on the atomically smooth substrate for layer deposition. Measurements were carried out after the complete evaporation of the solvent. A freshly cleaved surface of mica (SPI supplies, V-1 grade) was used as a substrate. The AFM measurements were carried out in the amplitude modulation tapping mode using 'RTESPA-150' type probes (Bruker, Billerica, Massachusetts, USA).

2.2. Experiments *in vivo*

The experimental animals (3-month-old male Wistar rats weighing 170 ± 5 g) were kept in an air-filtered and temperature-controlled (21 ± 1°C) room under 12-h light/12-h dark conditions [30]. They received water and a normal diet *ad libitum*. All procedures complied with the ARRIVE guidelines. The usage of the experimental animals was approved by the Biomedical Ethics Committee of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv (protocol No. 9 dated 4 September 2023) and performed following the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and Article 26 of the Law of Ukraine "On the Protection of Animals from Cruelty" (No. 3447-IV, 21 February 2006), as well as European Union Directive of 22 September 2010 (2010/63/EU) for the protection of animals used for scientific purposes.

The following groups of animals were used in the research (*n* = 10 in each): control, open injury, and open injury+C₆₀ (oral daily usage of C₆₀FAS in a dose of 1 mg/kg animal body weight after open muscle injury initiation).

The choice of the above dose of C₆₀FAS is based on its high effectiveness in the treatment of various muscle pathologies *in vivo* [25–27,30].

Anesthesia of experimental rats was performed by intraperitoneal injection of Nembutal (40 mg/kg). Animals were left under deep surgical anesthesia for at least 2 h [30].

Muscle gastrocnemius was isolated from surrounding tissues in the area of the hamstring fossa. This surgical procedure is described in detail in [30]. Then the *muscle gastrocnemius* was subjected to transverse dissection with a depth of 1 mm in three equidistant places (Figure 1) [31]. Such an open muscle injury model causes inflammation and necrosis of damaged tissues, as well as leads to significant muscle loss and its prolonged regeneration [32].

Electrical stimulation of the *muscle gastrocnemius* was performed by separate series of rectangular 2 ms pulses with a frequency of 50 Hz and a duration of 6 s using a strain gauge generator [30].

2.3. Biomechanical & biochemical analyses

Research of muscle contraction dynamics and blood biochemical indicators of rats was performed 5, 10, and 15 days after open muscle injury initiation.

The following biomechanical parameters [27], which are indicators of the overall performance of a muscle and have a clear tendency to change with an increase in its stiffness, were used:

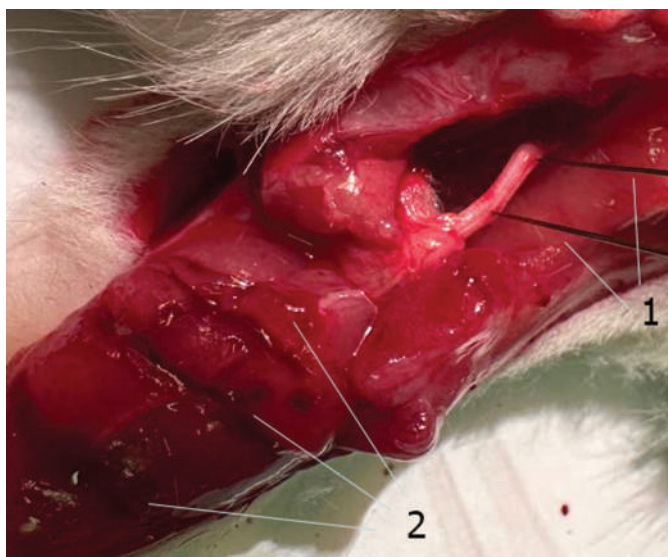


Figure 1. Open muscle injury model: 1 - stimulating electrodes; 2 - three transverse dissections with a depth of 1 mm in three equidistant places.

- time to reach the maximum force response of the muscle (t_{start}). The maximum force generation that an active muscle is capable of is an important indicator for fast non-targeted movements. The magnitude of its change indicates the level of physiological dysfunction of the neuromuscular preparation during the realization of maximal force tasks;
- time of retention of the maximum force response of the muscle (t_{max}). Its value is an indicator of the adaptability of the muscular system to new states resulting from the development of pathological processes. Changes in this parameter are associated with a decrease in the efficiency of individual muscle fibers, a decrease in their number, and, accordingly, an increase in the ratio of "muscle-fibrous tissue;"
- time of muscle contraction force return to the initial value after stimulation cessation (t_0). Its value is directly related to the amount of fibrous tissue formed in the injured muscle.

The following biochemical parameters, which are indicators of muscle injury [30], namely the concentrations of c-reactive protein (CRP), creatinine, lactate, and reduced glutathione (GSH) as well as the activity of catalase (CAT) and superoxide dismutase (SOD) in the blood plasma of rats were used. These parameters were measured using the biochemical analyzers RNL-200 and JN-1101-TR2 (Netherlands).

2.4. Histopathological analysis

For general histopathological analysis [33], the *muscle gastrocnemius* samples were separated and fixed in 10% formalin, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin (H&E) [34]. In addition, to detect connective tissue and muscle fibers, sections of the *muscle gastrocnemius* were stained with hematoxylin and picrofuchsin by van Gieson. Digital microphotographs of stained sections

($\times 100$ and $\times 400$ magnification) were taken using an Olympus Bx41 microscope and an Olympus C-5050 Zoom digital camera.

2.5. Statistical analysis

Statistical evaluation of the results was conducted using the software package Statistica 8.0 (Dell, USA) through the procedure of analysis of variances (ANOVA) with mixed design [30,34]. Two between-group factors were supposed: 1) open muscle injury (10 levels – 10 consecutive non-relaxation contractions of the *muscle gastrocnemius* when electrical stimulation is applied); 2) C_{60}FAS treatment (two levels – no and use of C_{60}FAS). Time was considered as a within-group factor with three levels (5, 10, and 15 days after initiation of open muscle injury). The Shapiro-Wilk W-test and Levene's test were used to assess for normality and the equality of variances across groups, respectively. The Bonferroni *post-hoc* test was used for multiple pairwise comparisons between different groups. The differences between the experimental groups were considered significant at $p < 0.05$. Each of the experimental force curves is the result of averaging 10 similar tests. Each biochemical measurement was carried out at least three times. Data are expressed as the means $\pm$ SEM for each group.

3. Results

3.1. C_{60}FAS characterization by AFM

When observing the process of deposition of C_{60} fullerene particles from C_{60}FAS on the mica surface, it was noted that the complete evaporation of water varied significantly in duration, ranging from 1 to 60 s within the area covered by the aqueous solution. This variation was accompanied by a significantly non-uniform distribution of the deposited substance over the surface. We carried out AFM measurements at locations with different surface concentrations of C_{60} fullerene.

The AFM image of the high-concentration area shows many clusters with heights ranging from 20 to 120 nm (Figure 2(a)), characteristic of colloidal C_{60}FAS . Such aggregates were observed rarely or completely absent in low-concentration areas. The AFM image of the low-concentration area reveals surface-spaced objects up to 3 nm in height (Figure 2(b)), which correspond to individual C_{60} fullerenes (diameter ~ 0.7 nm) or their nanoaggregates, which typically do not exceed four times the molecular diameter in size.

3.2. Biomechanics of injured muscle contraction

Figure 3 illustrates the results of 10 consecutive non-relaxation contractions of the *muscle gastrocnemius* of rats. As observed, during prolonged activation and the development of smooth tetanic muscle contraction, a decrease in the value of its force is evident. This is associated with alterations in the level of phosphorylation of myosin light chains, development of fatigue processes, a reduction in the number of active muscle fibers, inflammatory processes, and fibrosis of muscle tissue [13,24–27]. Based on the obtained mechanograms, the selective biomechanical parameters as indicators of muscle

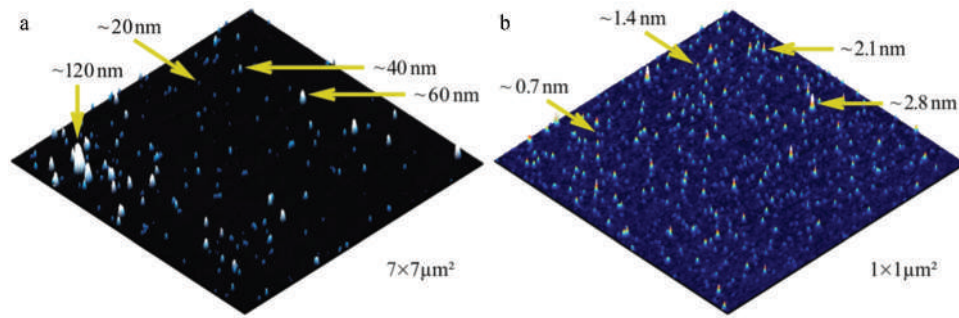


Figure 2. AFM images of C_{60} fullerene layer deposited from C_{60} FAS (0.15 mg/ml). The images were measured within one sample over different surface areas. The arrows indicate the height of the particles.

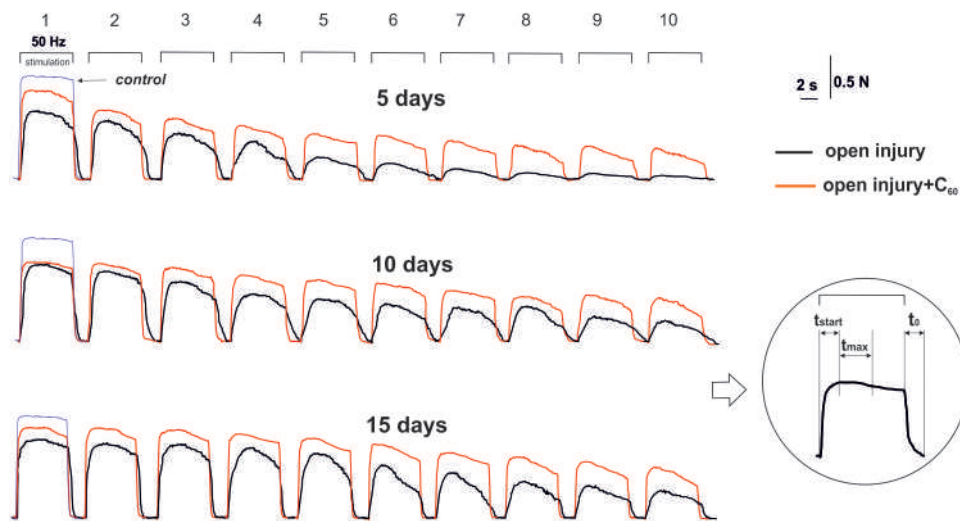


Figure 3. The force of 10 consecutive non-relaxation contractions of *muscle gastrocnemius* of rats (1–10) during application of 50 hz stimulation of 6 s duration: control, open injury and open injury+ C_{60} – mechanograms of a control group, groups of injured rats and rats who daily consumed orally C_{60} FAS at a dose of 1 mg/kg after the open muscle injury initiation; t_{start} – time to reach the maximum force response of the muscle; t_{max} – time of retention of the maximum force response of the muscle; t_0 – time of muscle contraction force return to the initial value after stimulation cessation; 5, 10 and 15 days – 5, 10 and 15 days after the open muscle injury initiation, respectively.

dysfunction (see below) were calculated at 5, 10, and 15 days following the open muscle injury initiation.

Figure 4 shows changes in the time to reach the maximum force response of the *muscle gastrocnemius* (t_{start}) during 10 consecutive non-relaxation contractions. Five days following the initiation of an open muscle injury, this index was 387 ± 8 ms at the 1st contraction (control – 97 ± 4 ms). At the 10th contraction, its value increased to 563 ± 11 ms. The application of C_{60} FAS significantly reduced this index, which was 143 ± 7 and 319 ± 9 ms at the 1st and 10th contraction, respectively. Consequently, the positive effect of C_{60} FAS was about 270% and 176% at the 1st and 10th contractions, respectively, compared to the open injury group.

On the 10th day after the open muscle injury initiation, the time to achieve the maximum force response of *muscle gastrocnemius* significantly increased and was 440 ± 12 and 680 ± 21 ms on the 1st and 10th contraction, respectively, which indicates an increase in connective tissue components in the studied muscle compared to the control group. In contrast, when C_{60} FAS was applied, this index remained practically at the same level as on the 5th day of the experiment, viz: $146 \pm$

5 and 331 ± 9 ms for the 1st and 10th contractions, respectively.

Mechanograms obtained on day 15 following the initiation of an open muscle injury demonstrate a decrease in the time to reach the maximal force response of the *muscle gastrocnemius* to 202 ± 7 and 401 ± 9 ms at the 1st and 10th contraction, respectively. However, these values exceeded the control ones by ~200% and ~400%, respectively. The application of C_{60} FAS reduced this value, which was 109 ± 3 (~12% relative to control) and 205 ± 5 (~112% relative to control) ms at the 1st and 10th contraction, respectively.

Figure 5 shows the results of changes in the retention time of the maximum force response of the *muscle gastrocnemius* (t_{max}) during 10 consecutive non-relaxation contractions. In the control group, the value of this parameter was maintained in all time frames of the experiment, with a value of 6 s. Five days after the open muscle injury initiation, this parameter was 2843 ± 12 ms at the 1st contraction and fell to almost zero at the 10th contraction. The application of C_{60} FAS increased it to 4720 ± 23 and 1090 ± 15 ms at the 1st and 10th contraction, respectively.

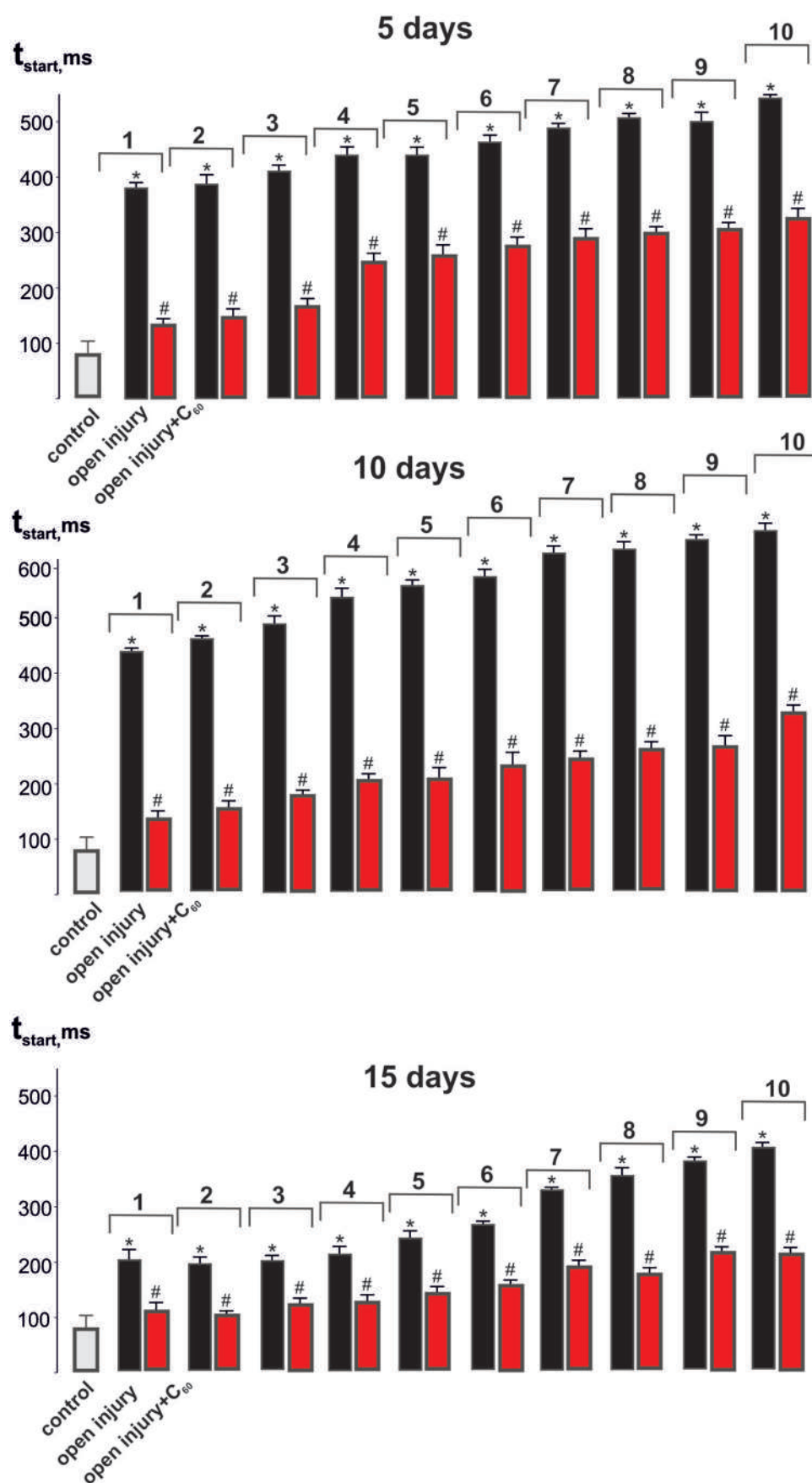


Figure 4. Time to reach the maximum force response of *muscle gastrocnemius* of rats (t_{start}) during 10 consecutive non-relaxation contractions (1–10) in the case of 50 Hz stimulation of 6 s duration: control, open injury, and open injury + C₆₀ – control group, group of injured rats and group of rats that daily consumed orally C₆₀ FAS at a dose of 1 mg/kg after the open muscle injury initiation; 5, 10 and 15 days – 5, 10 and 15 days after the open muscle injury initiation, respectively; * $p < 0.05$ relative to the control group; # $p < 0.05$ relative to the open injury group.

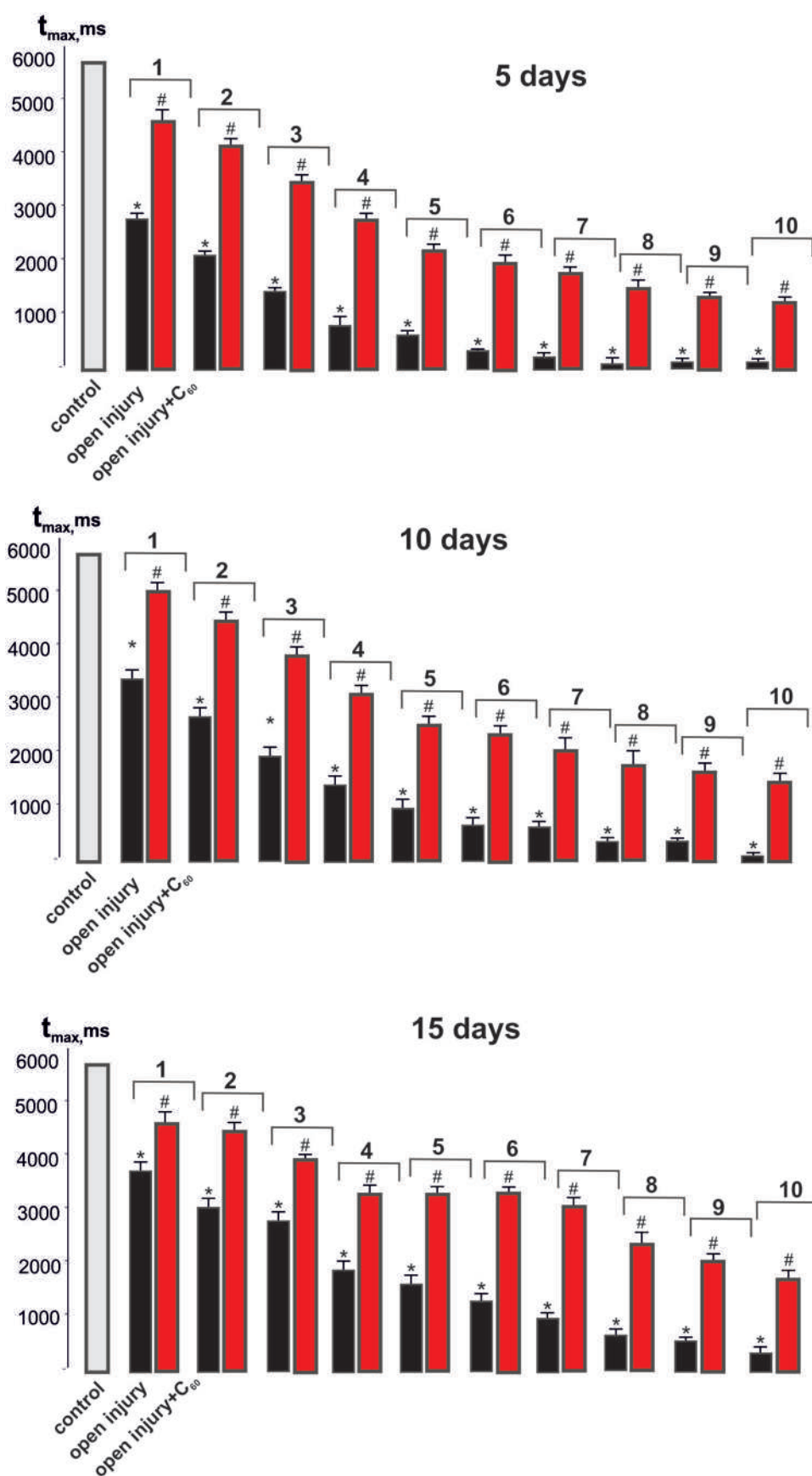


Figure 5. Time of retention of the maximum force response of the *muscle gastrocnemius* (t_{max}) during 10 consecutive non-relaxation contractions (1–10) in the case of 50 Hz stimulation of 6 s duration: control, open injury, and open injury + C₆₀ – control group, group of injured rats and group of rats that daily consumed orally C₆₀ FAS at a dose of 1 mg/kg after the open muscle injury initiation; 5, 10 and 15 days – 5, 10 and 15 days after the open muscle injury initiation, respectively; * $p < 0.05$ relative to the control group; # $p < 0.05$ relative to the open injury group.

On day 10 of the experiment, the value of this index decreased significantly to 3281 ± 14 ms at the 1st contraction and nearly reached zero at the 10th contraction. Application of C₆₀FAS increased in the retention time of the maximum force response of the *muscle gastrocnemius* to 5002 ± 3 and 1605 ± 5 ms at the 1st and 10th contraction, respectively.

On day 15 of the experiment, this indicator was 3943 ± 26 and 240 ± 12 ms at the 1st and 10th contractions, respectively. The application of C₆₀FAS in this case did not show significant changes compared with day 10 of the experiment, which was 4961 ± 24 and 1893 ± 17 ms at the 1st and 10th contractions, respectively.

Figure 6 shows the results of changes in the time of muscle contraction force return to its initial value after stimulation cessation (t_0) during 10 consecutive non-relaxation contractions of the *muscle gastrocnemius* of rats. 5 days after the open muscle injury initiation, this parameter was 1.32 ± 0.10 and 1.83 ± 0.10 s at the 1st and 10th contraction, respectively, with unchanged control values of 0.6 ± 0.1 s. The application of C₆₀FAS significantly reduced this parameter: 0.91 ± 0.10 and 1.09 ± 0.10 s at the 1st and 10th contraction, respectively.

On day 10 of the experiment, this parameter was 2.07 ± 0.20 and 2.81 ± 0.20 s at the 1st and 10th contractions, respectively, which was several hundred percent higher than the control value. Application of C₆₀FAS decreased this parameter, which was 1.24 ± 0.10 and 1.59 ± 0.10 s at the 1st and 10th contractions, respectively.

On the 15th day of the experiment, the time for the return of muscle contraction force to the initial value after stimulation cessation was 1.27 ± 0.10 and 1.69 ± 0.10 s at the 1st and 10th contraction, respectively, which was much higher than the control value. Application of C₆₀FAS reduced this parameter, which was 0.72 ± 0.10 (~43% relative to the open injury group) and 1.08 ± 0.10 s (~36% relative to the open injury group) at the 1st and 10th contraction, respectively.

3.3. Blood biochemical indicators of rats with injured muscle

CRP is produced by the liver and is a marker of inflammatory reactions in the body. The blood test for CRP shows the level of over-inflammatory processes in damaged muscle tissue, the concentration of which can increase 1000-fold in this case [35]. The increase of CRP concentration from 0.50 ± 0.04 mg/l in the control to 5.78 ± 0.20 mg/l after 5 days of the experiment is evidence of acute inflammatory processes in the damaged muscle (Figure 7). The slight decrease in its concentration to 5.23 ± 0.40 and 4.05 ± 0.30 mg/l on the 10th and 15th day of the experiment, respectively, indicates the presence of intense inflammatory processes occurring after the open muscle injury initiation throughout the experiment. The application of C₆₀FAS significantly reduced the value of this index, which was 3.95 ± 0.20 , 2.07 ± 0.20 , and 1.22 ± 0.10 mg/l on the 5th, 10th, and 15th day of the experiment, respectively.

The change in the concentration of creatinine, which is formed during the destruction of intramuscular structures, makes it possible to assess the degree of myocyte damage.

This index increased from 50 ± 2 μ M in the control to 225 ± 6 , 197 ± 4 , and 185 ± 5 μ M on the 5th, 10th, and 15th day of the experiment, respectively. Application of C₆₀FAS decreased its value to 188 ± 3 , 149 ± 5 , and 79 ± 2 μ M on the 5th, 10th, and 15th days of the experiment, respectively (Figure 7).

In control, the lactate level was 9.3 ± 0.3 mm. After the initiation of open muscle injury, its value increased to 17.4 ± 0.7 , 15.8 ± 0.8 , and 14.7 ± 0.6 mm on the 5th, 10th, and 15th day of the experiment, respectively. The application of C₆₀FAS decreased the lactate level to 12.1 ± 0.4 , 11.3 ± 0.5 , and 9.7 ± 0.3 mm on the 5th, 10th, and 15th day of the experiment, respectively (Figure 7).

SOD catalyzes the transformation reaction of free superoxide radicals into oxygen and hydrogen peroxide and is a key enzyme of the antioxidant system. The activity of SOD on days 5, 10, and 15 of the experiment was 8.7 ± 0.3 , 7.8 ± 0.4 , and 5.9 ± 0.3 Units/ml, respectively, with a control value of 1.9 ± 0.1 Units/ml. When C₆₀FAS was applied, these values decreased significantly and were 7.1 ± 0.3 , 4.4 ± 0.2 , and 2.9 ± 0.2 Units/ml on the 5th, 10th, and 15th day of the experiment, respectively (Figure 8).

CAT is a ferment that catalyzes the decomposition of hydrogen peroxide formed in the process of biological oxidation into water and molecular oxygen. CAT activity after the open muscle injury initiation increased from 0.8 ± 0.1 mm/min in the control to 5.4 ± 0.4 , 4.9 ± 0.4 , and 3.4 ± 0.2 mm/min on days 5, 10 and 15 of the experiment, respectively. Application of C₆₀FAS reduced this index to 4.5 ± 0.4 , 2.5 ± 0.2 , and 1.5 ± 0.1 mm/min on the 5th, 10th, and 15th day of the experiment, respectively (Figure 8).

Cellular mechanisms of antioxidant defense are also associated with the functioning of a powerful glutathione link [36]. The protective functions of GSH under oxidative stress are determined by its ability to catalyze the cleavage of hydrogen peroxide and fatty acid hydroperoxides. After the initiation of open muscle injury, the GSH concentration was 10.7 ± 1.0 , 9.2 ± 0.7 , and 7.9 ± 0.5 μ M on the 5th, 10th and 15th day of the experiment, respectively, while the control was 1.9 ± 0.2 μ M. After the application of C₆₀FAS, these values were 8.9 ± 0.5 , 6.8 ± 0.6 , and 3.7 ± 0.4 μ M on the 5th, 10th and 15th day of the experiment, respectively (Figure 8).

3.4. Histopathological analysis of the injured muscles

The *muscle gastrocnemius* of the control group has a normal histological structure. Muscle fibers are grouped in bundles, which are separated by thin layers of connective tissue. The connective tissue contains ground substance and collagen fibers (Figure 9(a)).

In animals with muscle injury, partially destroyed fibers are observed on the 15th day after initiation of the open muscle injury. Some of them are slightly altered, hypochromatic, with signs of edema, and hyperchromatic, with signs of destruction. Muscle fibers decrease in diameter, and their transverse striation is disrupted. The volume of connective tissue increases significantly. Fibrosis is observed. The content of the ground substance of connective tissue is also increased (Figure 9(b)).

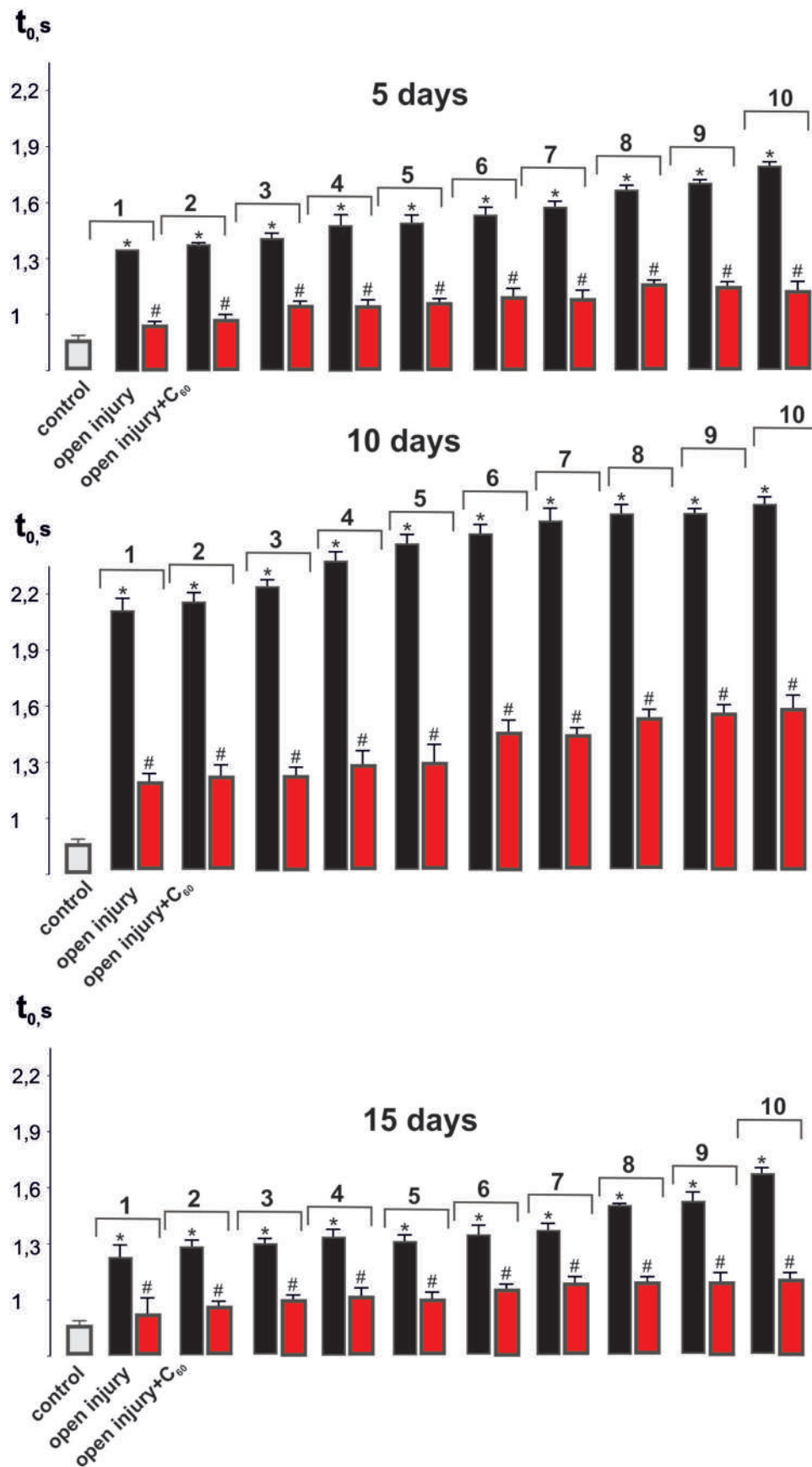


Figure 6. Time of muscle contraction force return to the initial value after stimulation cessation (t_0) during 10 consecutive non-relaxation contractions (1–10) in case of application of 50 hz stimulation of 6 s duration: control, open injury and open injury+C₆₀ – control group, group of injured rats and group of rats that daily consumed orally C₆₀FAS at a dose of 1 mg/kg after the open muscle injury initiation; 5, 10 and 15 days – 5, 10 and 15 days after the open muscle injury initiation, respectively; * $p < 0.05$ relative to the control group; # $p < 0.05$ relative to the open injury group.

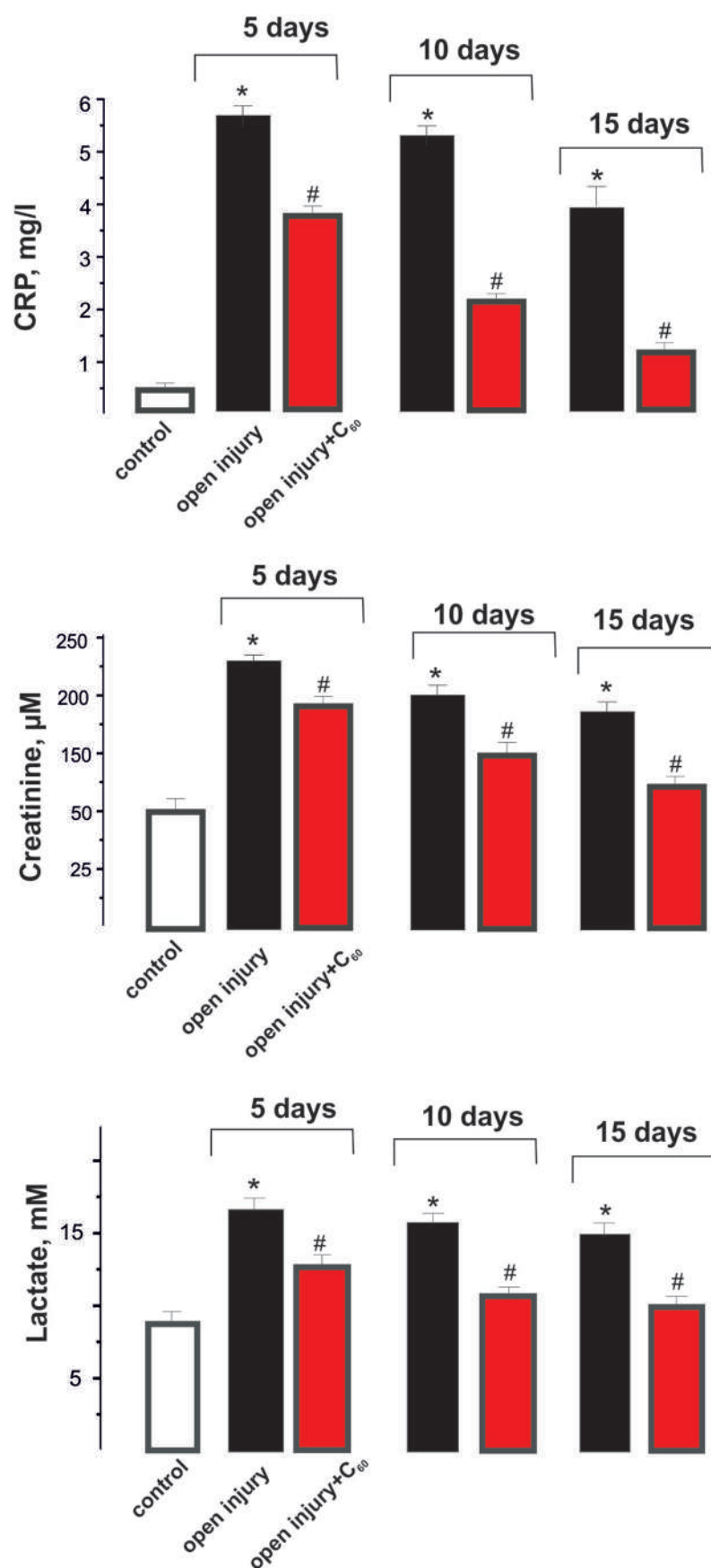


Figure 7. Concentrations of CRP, creatinine and lactate in the blood plasma of rats: control, open injury and open injury+C₆₀ - control group, group of injured rats and group of rats that daily orally administered C₆₀FAS at a dose of 1 mg/kg after the open muscle injury initiation; 5, 10 and 15 days – 5, 10 and 15 days after the open muscle injury initiation, respectively; * $p < 0.05$ relative to the control group; # $p < 0.05$ relative to the open injury group.

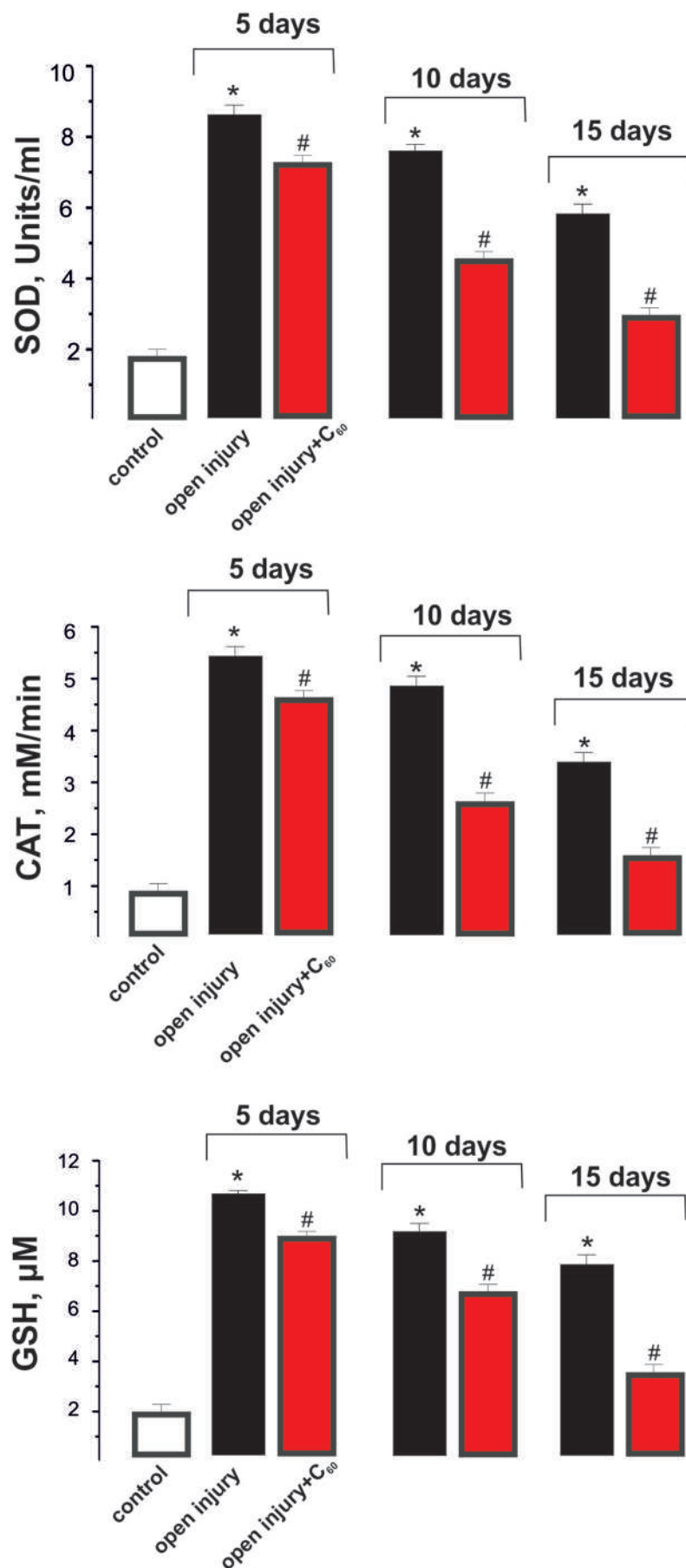


Figure 8. Indices of pro- and antioxidant balance (SOD, CAT and GSH) in the blood plasma of rats: control, open injury and open injury+C₆₀ - control group, group of injured rats and group of rats that daily consumed orally C₆₀FAS at a dose of 1 mg/kg after the open muscle injury initiation; 5, 10 and 15 days – 5, 10 and 15 days after the open muscle injury initiation, respectively; **p* < 0.05 relative to the control group; #*p* < 0.05 relative to the open injury group.

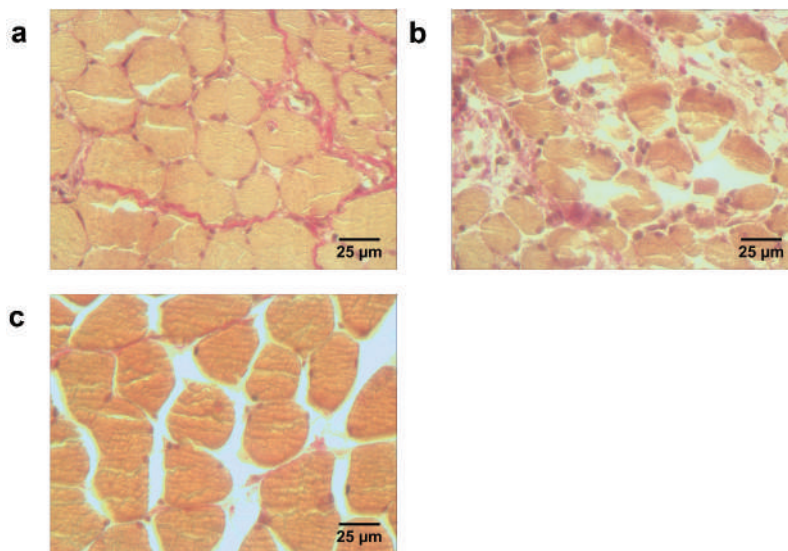


Figure 9. Histological images of the *muscle gastrocnemius*: a – control group; b – open muscle injury; c – open muscle injury+ C_{60} . Hematoxylin and picrofuchsin staining by van Gieson. Scale bars –25 μ m.

In animals with muscle injury, that received C_{60} FAS, the degree of pathohistological changes in the *muscle gastrocnemius* is lower: most of the fibers are slightly altered and some of them are hypochromatic, with the signs of edema. The connective tissue is increased, but less than in the previous experimental group. The increase in connective tissue occurred mainly due to the ground substance. Fibrosis is weakly expressed (Figure 9(c)).

4. Discussion

Since the biomedical applications of nanoparticles are highly dependent on their size [37], we performed a structural study of C_{60} FAS by AFM technique. It turned out that the studied C_{60} FAS is a polydisperse fluid, containing large C_{60} fullerene aggregates with a size of ~20–120 nm, and also individual C_{60} molecules (~0.7 nm) and C_{60} fullerene nanoaggregates with a size of ~1.4–2.8 nm, which is in good agreement with theoretical data [28]. Note that the zeta potential value of the resulting C_{60} FAS was -25 ± 2 mV [22]. A high negative charge of the colloidal particles indicates a low degree of aggregation over time. In summary, the detected predominantly nanoformation of the C_{60} fullerenes detected in an aqueous solution indicates their suitability for *in vivo* tests.

The dynamics of the beginning of the muscle contractile process are determined by subtle mechanisms of interaction between motoneuron pools with subsequent activation of the interaction between actin and myosin filaments. It is the stiffness of the muscle that determines its force of contraction. In other words, the dependence on “stimulation-contraction force” in the regeneration of damaged muscle is related to the level of fibrosis. Even small changes in the structure of passive elastic properties of muscle fibers lead to changes in the time of reaching the maximum muscle force response [13]. Note that the difference in the levels of the described positive effects of C_{60} FAS at the early and late stages of *muscle gastrocnemius* regeneration may be because, at the early stage (5 days after the initiation of

muscle injury), the post-traumatic inflammatory reaction with progressive necrosis of damaged muscle fibers is intensively occurring in muscle tissue [38]. Further use of C_{60} FAS promotes the inhibition of muscle fibrosis, which is consistent with the results [20] on the use of antioxidants against fibrosis formation in active muscle.

The time of retention of the maximum force response of the muscle or the stationary state of an active muscle is a temporary section of its force-contractile activity without a significant deviation to one side or the other during its activation. This parameter indicates the level of force production of the muscle, which corresponds to the physiological state of the neuromuscular preparation at a given time [39] and is an indicator of the course of repair processes in it. As can be seen, the effect of water-soluble C_{60} fullerenes on this index has a multifactorial character, in particular, the reduction of muscle stiffness and, as a consequence, fibrosis due to their antioxidant properties [16,20] and suppression of the course of the inflammatory process in closed muscle injuries [40].

The change in the time taken for the muscle contraction force to return to its initial value after stimulation cessation is directly influenced by the increase in the stiffness components of the active muscle [25]. While the results of the first two tests (on the 5th and 10th day of the experiment) can be explained by the suppression of inflammatory processes by the antioxidant properties of C_{60} fullerenes, on the 15th day of the experiment inflammatory processes do not play a key role in the increase of muscle stiffness [11,41]. The results obtained suggest a potential relationship between C_{60} fullerenes and the reduction of fibrosis and, as a consequence, muscle stiffness in the process of its post-traumatic repair. The interaction of different cell types during muscle injury repair is supported by numerous factors including proteolytic enzymes, growth factors, angiogenic factors, and fibrogenic cytokines, which together stimulate connective tissue formation. However, there are still many unknown initiators and fibrotic pathway participants, and significant differences between tissue systems. At the same time, experimental evidence suggests that post-traumatic fibrosis is associated with redox imbalance [42],

and NADPH oxidase may be the most important link between redox imbalance, free radical production, and fibrosis [43]. It has also been shown that maintaining redox balance may be an effective therapeutic strategy for fibrotic disorders [20,44], and that reducing muscle stiffness may influence its post-traumatic biomechanics.

In summary, many studies have addressed the problem of finding effective therapeutic methods to reduce fibrosis formation. For example, analysis of quantitative muscle stiffness using shear wave elastography shows that fibrosis is reduced in a porcine model of volumetric muscle loss injury by applying nintedanib as an antifibrotic agent [45]. Losartan administration reduces fibrosis but hinders the functional recovery of rat muscle after injury [46]. Finally, quercetin attenuates fibrosis in human skeletal muscle [20].

The analysis of blood biochemical parameters at muscle injury and its further post-traumatic recovery provides an opportunity to evaluate the effectiveness of the applied therapeutic drug. In the present study, we used biochemical indices of inflammation levels (CRP) and fatigue process development (creatinine and lactate), which serve as markers of physiological disturbances in active muscle due to injury. It was found that the application of C₆₀FAS significantly decreased the CRP value and the creatinine fraction, indicating the promise of using antioxidants in the therapy of muscle inflammation [47].

In active muscle, many metabolic processes take place in anaerobic conditions, so it uses a large number of mitochondrial ferments and, as a result, it accumulates a large amount of lactate, which does not have time to be oxidized during its prolonged stimulation. Thus, the proposed antioxidant therapy led to an increase in lactate oxidation, and at the end of the experiment, its concentration approached the control.

Pathological inflammatory processes that occur immediately following muscle injury are a source of free oxygen radicals and contribute to the intensification of lipid peroxidation processes [48]. Therefore, in the present study, we evaluated changes in the blood of experimental animals in biochemical parameters – markers of peroxidation and oxidative stress (SOD, CAT, and GSH) during exposure to C₆₀FAS. It was shown that the long-term application of water-soluble C₆₀ fullerenes, as powerful antioxidants, helps mitigate oxidative processes in damaged muscles. This was achieved by maintaining the balance between prooxidants and the antioxidant defense system, thereby preventing the negative effect of free radicals on cellular and subcellular structures during the post-traumatic recovery of rat skeletal muscles.

Finally, the biomechanical and biochemical results obtained were confirmed by the histopathological analysis: in animals, that received C₆₀FAS (open muscle injury+C₆₀ group), the degree of pathohistological changes in the *muscle gastrocnemius* was less than in the open muscle injury group; specifically, fibrosis was weakly expressed.

5. Conclusions

Based on the above biomechanical and biochemical data, we can conclude that the application of nanoantioxidants C₆₀ fullerenes within 15 days (daily oral dose 1 mg/kg) after the onset of open injury of the *muscle gastrocnemius* reduces its stiffness and, as a consequence, inhibits the development of muscle fibrosis, as well as reduces the intensity of

inflammatory processes in the body. Histopathological data confirm these results. By influencing the activity of endogenous antioxidants, C₆₀ fullerenes can suppress the formation of significant destruction in injured muscles. Therefore, they can be considered potent nanoagents for the correction of muscle damage that occurs during its physiological repair, which requires further clinical studies.

CRediT authorship contribution statement

Yuriy Prylutskyi: Conceptualization, Writing – review & editing, Supervision. **Dmytro Nozdrenko:** Investigation, Methodology, Writing – review & editing. **Olexandr Motuziuk:** Investigation, Methodology, Formal analysis. **Svitlana Prylutska:** Investigation, Methodology. **Igor Vareniuk:** Investigation. **Natalia Nurishchenko:** Investigation. **Daria Franskevych:** Investigation. **Vasil Soroca:** Investigation. **Kateryna Bogutska:** Formal analysis, Writing – original draft preparation. **Uwe Ritter:** Writing – review and editing, Project administration.

Disclosure statement

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Funding

This research was supported by the National Research Foundation of Ukraine [2022.01/0004].

Ethics Disclosure

All of the *in vivo* experiments were approved by the Bioethics Committee of the ESC “Institute of Biology and Medicine” of Taras Shevchenko National University of Kyiv (protocol No. 9 dated September 4, 2023) in accordance with the norms of biomedical ethics in accordance with the Law of Ukraine No. 3447-IV of 21.02.2006, Kyiv, “On the Protection of Animals from Cruelty” as well as the European Union Directive (2010/63/EU) “On the protection of animals used for scientific purposes”.

ORCID

Yuriy Prylutskyi  <http://orcid.org/0000-0002-9847-4137>
Dmytro Nozdrenko  <http://orcid.org/0000-0001-7254-1533>
Olexandr Motuziuk  <http://orcid.org/0000-0002-1520-0721>
Svitlana Prylutska  <http://orcid.org/0000-0001-5280-8341>
Igor Vareniuk  <http://orcid.org/0000-0002-9881-5536>
Natalia Nurishchenko  <http://orcid.org/0000-0001-8160-8832>
Daria Franskevych  <http://orcid.org/0000-0002-1719-0512>
Vasil Soroca  <http://orcid.org/0000-0002-8340-7636>
Kateryna Bogutska  <http://orcid.org/0000-0003-0202-8042>
Uwe Ritter  <http://orcid.org/0000-0002-9315-4863>

References

Papers of special note have been highlighted as either of interest (*) or of considerable interest () to readers.**

1. Blackburne LH. Combat damage control surgery. *Crit Care Med.* 2008;36:S304–310. doi: 10.1097/CCM.0b013e31817e2854

2. Sakorafas GH, Peros G. Principles of war surgery: current concepts and future perspectives. *Am J Emerg Med*. 2008;26:480–489. doi: [10.1016/j.ajem.2007.05.009](#)
3. Eryilmaz M, Uzar AI. War surgery in the 21st century: current approach to trauma cases. *Ulus Travma Acil Cerrahi Derg*. 2008;14:268–276.
4. Hoogervorst P, Shearer DW, Miclau T. The burden of high-energy musculoskeletal trauma in high-income countries. *World J Surg*. 2020;44:1033–1038. doi: [10.1007/s00268-018-4742-3](#)
5. Hamilton B, Valle X, Rodas G, et al. Classification and grading of muscle injuries: a narrative review. *Br J Sports Med*. 2015;49:306. doi: [10.1136/bjsports-2014-093551](#)
6. Paffrath T, Lefering R, Flohé S, et al. How to define severely injured patients? – an Injury Severity Score (ISS) based approach alone is not sufficient. *Injury*. 2014;45:S64–69. doi: [10.1016/j.injury.2014.08.020](#)
7. Kulla M, Maier J, Bieler D, et al. Civilian blast injuries: an underestimated problem? Results of a retrospective analysis of the TraumaRegister DGU®. *Unfallchirurg*. 2016;119:843–853. doi: [10.1007/s00113-015-0046-3](#)
8. Champion HR, Holcomb JB, Young LA. Injuries from explosions: physics, biophysics, pathology, and required research focus. *J Trauma*. 2009;66:1468–1477. doi: [10.1097/TA.0b013e3181a27e7f](#)
9. Ebrahimi A, Nejadsharvari N, Ebrahimi A, et al. Early reconstructions of complex lower extremity battlefield soft tissue wounds. *World J Plast Surg*. 2017;6:332–342.
10. Connolly M, Ibrahim ZR, Johnson ON 3rd. Changing paradigms in lower extremity reconstruction in war-related injuries. *Mil Med Res*. 2016;3:9. doi: [10.1186/s40779-016-0080-7](#)
11. Mann CJ, Perdiguer E, Kharraz Y, et al. Aberrant repair and fibrosis development in skeletal muscle. *Skelet Muscle*. 2011;1:21. doi: [10.1186/2044-5040-1-21](#)
- **Fibrosis development in skeletal muscle.**
12. Westman AM, Peirce SM, Christ GJ, et al. Agent-based model provides insight into the mechanisms behind failed regeneration following volumetric muscle loss injury. *PLoS Comput Biol*. 2021;17:e1008937. doi: [10.1371/journal.pcbi.1008937](#)
13. Mahdy MAA. Skeletal muscle fibrosis: an overview. *Cell Tissue Res*. 2019;375:575–588. doi: [10.1007/s00441-018-2955-2](#)
- **Skeletal muscle fibrosis.**
14. Moyer AL, Wagner KR. Regeneration versus fibrosis in skeletal muscle. *Curr Opin Rheumatol*. 2011;23:568–573. doi: [10.1097/BOR.0b013e31823834bac92](#)
15. Serrano AL, Muñoz-Cánoves P. Regulation and dysregulation of fibrosis in skeletal muscle. *Exp Cell Res*. 2010;316:3050–3058. doi: [10.1016/j.yexcr.2010.05.035](#)
- **Fibrosis development in skeletal muscle.**
16. Kozakowska M, Pietraszek-Gremplewicz K, Jozkowicz A, et al. The role of oxidative stress in skeletal muscle injury and regeneration: focus on antioxidant enzymes. *J Muscle Res Cell Motil*. 2015;36:377–393. doi: [10.1007/s10974-015-9438-9](#)
- **Antioxidant therapy for muscle injuries.**
17. Hsu YJ, Ho CS, Lee MC, et al. Protective effects of resveratrol supplementation on contusion induced muscle injury. *J Med Sci*. 2020;17:53–62. doi: [10.7150/ijms.35977](#)
18. Lee JE, Shah VK, Lee EJ, et al. Melittin – a bee venom component – enhances muscle regeneration factors expression in a mouse model of skeletal muscle contusion. *J Pharmacol Sci*. 2019;140:26–32. doi: [10.1016/j.jphs.2019.03.009](#)
19. Hirose J, Yamaga M, Kato T, et al. Effects of a hydroxyl radical scavenger, EPC-K1, and neutrophil depletion on reperfusion injury in rat skeletal muscle. *Acta Orthop Scand*. 2001;72:404–410. doi: [10.1080/000164701753542078](#)
20. Ohmae S, Akazawa S, Takahashi T, et al. Quercetin attenuates adipogenesis and fibrosis in human skeletal muscle. *Biochem Biophys Res Commun*. 2022;615:24–30. doi: [10.1016/j.bbrc.2022.05.033](#)
21. Gharbi M, Pressac M, Hadchouel M, et al. [60]fullerene is a powerful antioxidant *in vivo* with no acute or subacute toxicity. *Nano Lett*. 2005;5:2578–2585. doi: [10.1021/nl051866b](#)
- **C60fullerene as a powerful antioxidant.**
22. Prylutska SV, Grebinyk AG, Lynchak OV, et al. *In vitro* and *in vivo* toxicity of pristine C₆₀ fullerene aqueous colloid solution. *Fullerenes Nanotubes Carbon Nanostructures*. 2019;27:715
- **In vitro and in vivo toxicity of C60 fullerene.**
23. Eswaran SV. Water soluble nanocarbon materials: a panacea for all? *Curr Sci*. 2018;114:1846–1850. doi: [10.18520/cs/v114/i09/1846-1850](#)
24. Nozdrenko D, Prylutska S, Bogutska K, et al. Analysis of biomechanical and biochemical markers of rat *muscle soleus* fatigue processes development during long-term use of C₆₀ fullerene and N-acetylcysteine. *Nanomater*. 2022;12:1552.
25. Nozdrenko D, Matvienko T, Vygovska O, et al. Protective effect of water-soluble C₆₀ fullerene nanoparticles on the ischemia-reperfusion injury of the muscle soleus in rats. *Int J Mol Sci*. 2021;22:6812. doi: [10.3390/ijms22136812](#)
26. Nozdrenko D, Prylutska S, Bogutska K, et al. Effect of C₆₀ fullerene on recovery of muscle soleus in rats after atrophy induced by achillectomy. *Life*. 2022;12:332. doi: [10.3390/life12030332](#)
27. Nozdrenko D, Motuziuk O, Prylutska S, et al. C₆₀ fullerene protective effect against the rat muscle soleus trauma. *Heliyon*. 2024;11:e32677. doi: [10.1016/j.heliyon.2024.e32677](#)
28. Prilutski Y, Durov S, Bulavin L, et al. Study of structure of colloidal particles of fullerenes in water solution. *Mol Cryst Liq Cryst*. 1998;324:65–70.
29. Skivka LM, Prylutska SV, Rudyk MP, et al. C₆₀ fullerene and its nanocomplexes with anticancer drugs modulate circulating phagocyte functions and dramatically increase ROS generation in transformed monocytes. *Cancer Nanotechnol*. 2018;9:8. doi: [10.1186/s12645-017-0034-0](#)
30. Prylutska Y, Nozdrenko D, Motuziuk O, et al. C₆₀ fullerene improves the contractile activity of the injured rat *muscle gastrocnemius*. *Nanotechnol*. 2025;36. doi: [10.1088/1361-6528/ada29a](#)
31. Sicherer ST, Venkatarama RS, Grasman JM. Recent trends in injury models to study skeletal muscle regeneration and repair. *Bioengineering*. 2020;7:76. doi: [10.3390/bioengineering7030076](#)
32. Corona BT, Ward CL, Baker HB, et al. Implantation of *in vitro* tissue engineered muscle repair constructs and bladder acellular matrices partially restore *in vivo* skeletal muscle function in a rat model of volumetric muscle loss injury. *Tissue Eng Part A*. 2014;20:705–715. doi: [10.1089/ten.TEA.2012.0761](#)
33. Suvana KS, Layton C, Bancroft JD. Bancroft's theory and practice of histological techniques. 8th (Elsevier Health Sciences) ed. 2018. p. 672.
34. Prylutska Y, Nozdrenko D, Omelchuk O, et al. Effect of C₆₀ fullerene on muscle injury-induced rhabdomyolysis and associated acute renal failure. *Int J Nanomed*. 2024;19:8043–8058. doi: [10.2147/IJN.S468013](#)
35. Sproston NR, Ashworth JJ. Role of C-reactive protein at sites of inflammation and infection. *Front Immunol*. 2018;9:754. doi: [10.3389/fimmu.2018.00754](#)
36. Sen CK. Antioxidant and redox regulation of cellular signaling: introduction. *Med Sci Sports Exerc*. 2001;33:368–370. doi: [10.1097/00005768-200103000-00005](#)
37. Abbasi R, Shineh G, Mobaraki M, et al. Structural parameters of nanoparticles affecting their toxicity for biomedical applications: a review. *J Nanopart Res*. 2023;25:43. doi: [10.1007/s11051-023-05690-w](#)
38. Grassi A, Quaglia A, Canata GL, et al. An update on the grading of muscle injuries: a narrative review from clinical to comprehensive systems. *Joints*. 2016;4:39–46. doi: [10.11138/jts/2016.4.1.039](#)
39. Kostyukov AI. Theoretical analysis of the force and position synergies in two-joint movements. *Neurophysiology*. 2016;48:287–296. doi: [10.1007/s11062-016-9601-y](#)
40. Nozdrenko D, Matvienko T, Vygovska O, et al. Post-traumatic recovery of *muscle soleus* in rats is improved via synergistic effect of C₆₀ fullerene and TRPM8 agonist menthol. *Appl Nanosci*. 2022;12:467–478. doi: [10.1007/s13204-021-01703-z](#)
- **C60fullerene for muscle injuries.**
41. Kharraz Y, Guerra J, Mann CJ, et al. Macrophage plasticity and the role of inflammation in skeletal muscle repair. *Mediators Inflamm*. 2013;2013:491497. doi: [10.1155/2013/491497](#)

42. Hecker L, Logsdon NJ, Kurundkar D, et al. Reversal of persistent fibrosis in aging by targeting Nox4-Nrf2 redox imbalance. *Sci Transl Med.* 2014;6:231ra47. doi: [10.1126/scitranslmed.3008182](https://doi.org/10.1126/scitranslmed.3008182)
43. Chan EC, Peshavariya HM, Liu GS, et al. Nox4 modulates collagen production stimulated by transforming growth factor β 1 *in vivo* and *in vitro*. *Biochem Biophys Res Commun.* 2013;430:918–925. doi: [10.1016/j.bbrc.2012.11.138](https://doi.org/10.1016/j.bbrc.2012.11.138)
44. Wu Y, Wu Y, Yu J, et al. Irisin ameliorates D-galactose-induced skeletal muscle fibrosis via the PI3K/Akt pathway. *Eur J Pharmacol.* 2023;939:175476. doi: [10.1016/j.ejphar.2022.175476](https://doi.org/10.1016/j.ejphar.2022.175476)
45. Corona BT, Rivera JC, Dalske KA, et al. Pharmacological mitigation of fibrosis in a porcine model of volumetric muscle loss injury. *Tissue Eng Part A.* 2020;26(11–12):636–646. doi: [10.1089/ten.TEA.2019.0272](https://doi.org/10.1089/ten.TEA.2019.0272)
46. Garg K, Corona BT, Walters TJ. Losartan administration reduces fibrosis but hinders functional recovery after volumetric muscle loss injury. *J Appl Physiol.* 2014;117(10):1120–1131. doi: [10.1152/japplphysiol.00689.2014](https://doi.org/10.1152/japplphysiol.00689.2014)
47. Duchesne E, Dufresne SS, Dumont NA. Impact of inflammation and anti-inflammatory modalities on skeletal muscle healing: from fundamental research to the clinic. *Phys Ther.* 2017;97:807–817. doi: [10.1093/ptj/pzx056](https://doi.org/10.1093/ptj/pzx056)
48. Cuzzocrea S, Riley DP, Caputi AP, et al. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. *Pharmacol Rev.* 2001;53:135–159.

Research Article

C₆₀ Fullerenes Diminish Dysfunctions in Rat *Muscle Soleus* With Chronic Alcohol Intoxication

Olexandr Motuziuk ^{1,2}, Dmytro Nozdrenko ¹, Svitlana Prylutska ³, Yurii Osyp ²,
Kateryna Bogutska ¹, Olena Dmytrotsa ², Yuriy Prylutskyi ¹, Peter Scharff ⁴,
and Uwe Ritter ⁴

¹Department of Biophysics and Neurobiology, ESC “Institute of Biology and Medicine”,
Taras Shevchenko National University of Kyiv, Kyiv 01601, Ukraine

²Department of Human and Animal Physiology, Faculty of Biology and Forestry, Lesya Ukrainka Volyn National University,
Lutsk 43025, Ukraine

³Department of Physiology, Plant Biochemistry and Bioenergetics, Faculty of Plant Protection, Biotechnology and Ecology,
National University of Life and Environmental Science of Ukraine, Kyiv 03041, Ukraine

⁴Institute of Chemistry and Biotechnology, Technical University of Ilmenau, Ilmenau 98693, Germany

Correspondence should be addressed to Uwe Ritter; uwe.ritter@tu-ilmenau.de

Received 15 January 2025; Revised 5 May 2025; Accepted 4 June 2025

Academic Editor: Rajaram Rajamohan

Copyright © 2025 Olexandr Motuziuk et al. Journal of Nanotechnology published by John Wiley & Sons Ltd. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

It is known that reactive oxygen species are important in the development of alcoholic myopathy, and their suppression by C₆₀ fullerene nanoparticles, as potent antioxidants, is opening up a new direction for their use in biomedical nanotechnology. Here, for the first time, the biomechanical and biochemical parameters of the functioning of rat *muscle soleus* under alcohol intoxication at 3, 6, and 9 months were analyzed. C₆₀ fullerene, as a therapeutic nanoagent, at an oral dose of 1 mg kg⁻¹ was used along with alcohol throughout the experiment. The findings indicate that significant dysfunctions develop in the rat *muscle soleus* after chronic alcohol intoxication. However, these dysfunctions are significantly reduced due to exposure to C₆₀ fullerenes, particularly with prolonged alcoholization of animals (9 months) and the development of high-intensity fatigue processes.

Keywords: alcohol intoxication; biomechanical parameters of muscle contraction; C₆₀ fullerene; *muscle soleus*; pro- and anti-oxidant balance in muscle tissue

1. Introduction

Alcoholic myopathy is characterized by biochemical, physiological, and structural changes in skeletal muscles leading to a disorder of their functional activity [1]. This pathology is most pronounced in the fast-contracting muscles [2] and is characterized by progressive proximal weakness, pain, atrophy, and impaired mobility. The results of studies [3–5] show that chronic alcohol consumption causes a decrease in the mass of fast muscles and their physiological cross-sectional area. In contrast, the decrease in the cross-sectional area of slow fibers is insignificant

under similar conditions of alcoholization [6]. Alcohol consumption for 14–16 weeks correlates with a decrease in maximal muscle tension and tetanic strength of fast muscles. In addition, in alcohol-fed mice, fast muscles developed significantly lower contraction force during high-frequency stimulation compared to slow muscles [5]. Chronic alcohol consumption also reduces the time to onset of muscle fatigue [7, 8].

The development of alcoholic myopathy increases the level of oxidative stress in the body. In addition to significant atrophy, the fast muscles of alcohol-fed rats exhibited a decrease in glutathione (GSH) levels, the activity of GSH

reductase, superoxide dismutase (SOD), and glutathione peroxidase (GP_x), as well as an increase in NADPH Oxidase-1 gene expression, indicating significant oxidative stress [9]. The authors [10] found high levels of cholesterol hydroperoxides in the tibialis anterior muscle (a fast muscle) compared to the vastus lateralis (a slow muscle), suggesting low antioxidant activity in alcohol-exposed muscles rich in Type II fibers [11]. However, by using the increased concentration of carbonyls as indicators of muscle protein damage caused by reactive oxygen species (ROS), the authors [12] found that Type II muscles exhibit a higher protective effect than Type I muscles during chronic alcoholization.

The use of the antioxidant procysteine attenuated the atrophy of alcoholic fast skeletal muscle, restored GSH levels, and increased catalase (CAT) mRNA expression but did not reduce the levels of other markers of oxidative stress and catabolic disorders. It is hypothesized that it minimizes muscle atrophy by inducing components of several anabolic pathways [9]. The use of antioxidants, such as vitamins C and E, exerted different effects on the two types of alcohol-exposed muscle tissue [13]. For example, in Type II muscle fibers, the effectiveness of antioxidant defense mechanisms in balancing increased ROS production was reduced, making them more susceptible to oxidative stress. This, in turn, led to impaired muscle contractile function, rapid fatigue, and injury.

It is known that biocompatible and bioavailable carbon nanoparticles such as C₆₀ fullerenes [14] with antioxidant properties can effectively inactivate ROS in living systems [15–17]. Recent studies [18, 19] have demonstrated that the level of chronic alcoholic myopathy development in rat *muscle gastrocnemius* (a fast skeletal muscle) decreases when C₆₀ fullerene is used as a therapeutic nanoagent. It was found that C₆₀ fullerene reduces the level of liver damage in chronic alcohol intoxication of rats [20]. However, there is currently no data on the effect of this nanoantioxidant on the functional activity of slow skeletal muscle (*muscle soleus*) in rats during alcoholic myopathy.

Thus, the novelty of the current research lies in assessing the biomechanical and biochemical parameters of the functioning rat *muscle soleus* under chronic alcohol intoxication and exposure to C₆₀ fullerenes over periods of 3, 6, and 9 months.

2. Materials and Methods

2.1. Sample Preparation and Characterization. A previously proposed ultrasonic method [21] was used to prepare a highly stable (for 18 months at a temperature of +4°C–25°C) C₆₀ fullerene aqueous solution (C₆₀FAS; initial concentration 0.15 mg mL^{−1}) [20].

The structural organization of C₆₀ fullerene particles in aqueous solution was investigated using atomic force microscopy (AFM; NT-MDT, Apeldoorn, Netherlands) with “RTESPA-150” probes (Bruker, Billerica, Massachusetts, USA) [20].

2.2. In Vivo Experiments. The research was conducted on male Wistar rats aged 1 month (at the beginning of the experiment) with a body weight of 170 ± 10 g [20]. The rats were housed under controlled environmental conditions (21°C, 12 h light–12 h dark cycle) with free access to water and a standard diet *ad libitum* [22]. All procedures with laboratory animals complied with the ARRIVE guidelines. The use of animals was approved by the Biomedical Ethics Committee of the ESC “Institute of Biology and Medicine” at Taras Shevchenko National University of Kyiv (protocol no. 2 dated September 2, 2022) and conducted in accordance with Article 26 of the Law of Ukraine “On the Protection of Animals from Cruelty” (no. 3447-IV, 21.02.2006), as well as European Union Directive of 22 September 2010 (2010/63/EU) for the protection of animals used for scientific purposes.

The laboratory animals were randomly divided into the following experimental groups:

- Intact group ($n = 30$): Rats received 100% drinking water;
- Alcoholization group (control; $n = 30$): Each rat was placed in a separate cage and provided with alcohol (40% ethanol in drinking water). This meant that the animals did not have access to 100% water until they had consumed the dosed portion of ethanol [23]. The amount of ethanol administered was calculated as 0.5% of the animal's body weight. The ethanol dose was recalculated every 24 h throughout the experiment. The duration of alcoholization was 3 ($n = 10$), 6 ($n = 10$), and 9 ($n = 10$) months.

The target concentration of 40% ethanol in drinking water was chosen because it reflects the blood alcohol concentration (BAC) reported in chronic alcoholics [24]. The BAC of the animals was determined using an alcohol analyzer (AM1, UK).

It should be noted that the rat model of chronic alcoholization for more than 9 months demonstrates the development of irreversible multiple organ failure [25], which was the criterion for choosing the upper limit of the duration of animal alcohol intoxication;

- Alcoholization + C₆₀ ($n = 30$; 10 animals in each subgroup): These rats were administered C₆₀FAS at a daily oral dose of 1 mg kg^{−1} along with alcohol. The C₆₀FAS dose was recalculated every 24 h throughout the experiment. Control over the amount of C₆₀FAS ingested was ensured by denying the rats' access to drinking 100% water until they fully consumed the administered dose.

It should be noted that the dose of C₆₀FAS used in this study was chosen as the most effective dose based on previously obtained results [18–20]. Additionally, this value is significantly lower than the LD₅₀, which was found to be 600 mg kg^{−1} body weight when administered orally to rats [26].

Rats were anesthetized via intraperitoneal injection of Nembutal (40 mg kg^{-1}), maintaining deep surgical anesthesia for at least 2 h [27]. The target skeletal muscles were isolated from surrounding tissues, tendons were transected distally, and ventral roots were severed at their spinal cord exit points [22, 27].

2.3. Biomechanical and Biochemical Analyses. The mechanokinetic parameters of muscle contraction were recorded using a National Instruments (USA) USB-6009, a 12-bit analog-to-digital converter (ADC) [27].

The following biomechanical parameters of rat skeletal muscle function, serving as markers of dysfunctions [19], were analyzed:

- Time to establish smooth tetanic muscle contraction (t_1);
- Time for contraction force to decay to zero after stimulation cessation (t_2);
- Integrated muscle power (area under the force curve; S) calculated numerically using Origin 9.4 software;
- The minimum force during a single contractile act ($F_{\min}$);
- Time to reach peak force response (t_{start}).

The pro- and antioxidant balance in muscle tissue, assessed by measuring reduced GSH level and the activities of selenium-dependent GP_x, CAT, and SOD, was evaluated as markers of muscle damage [22, 27, 28]. Analyses were performed using biochemical analyzers: RNL-200 (Netherlands), ABX Micros ESV60, and Pentra C400 (France).

2.4. Statistical Analysis. The statistical analysis of the experimental results was conducted using the software package Statistica 8.0 (Dell, USA) through a mixed design analysis of variance (ANOVA) procedure [27]. Two between-group factors were considered: (1) alcoholization (with three levels: 3, 6, and 9 months of alcohol exposure) and (2) C₆₀FAS treatment (with two levels: no treatment and C₆₀FAS treatment). The Shapiro–Wilk W-test and Levene's test were used to assess the normality and equality of variances across groups, respectively. Multiple pairwise comparisons between different groups and conditions were performed by the Bonferroni *post hoc* test. Differences between groups were considered statistically significant at $p < 0.05$. Each experimental force curve represents the average of 10 similar tests, and all biochemical measurements were repeated at least three times.

3. Results and Discussion

3.1. AFM Study. Since the size of nanoparticles affects their toxicity for biomedical applications [29, 30], we conducted AFM research on the resulting C₆₀FAS.

As seen in the 3D AFM image (Figure 1), the C₆₀FAS consisted of randomly located point-like objects, with heights reaching up to 3.5 nm. The typical width of their profiles was 10–15 nm. Taking into account that the radius of

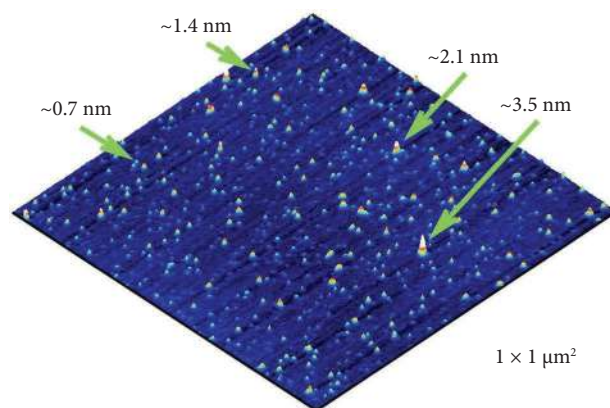


FIGURE 1: 3D AFM image of C₆₀ fullerenes deposited from C₆₀FAS (0.15 mg mL^{-1}) on a mica substrate. The arrows indicate the height of the observed nanoparticles.

curvature of the tip of the used AFM probe was $\sim 12 \text{ nm}$, we believe that the nanoobjects had a spherical shape, corresponding to either a single C₆₀ molecule ($\sim 0.7 \text{ nm}$) or its nanoaggregates with diameters ranging from 1.4 to 3.5 nm. This was consistent with our previous theoretical results [31].

Thus, the detected nanoformation of the C₆₀ fullerenes in an aqueous solution indicates their suitability for *in vivo* tests.

3.2. Muscle Contraction Analysis. Figures 2(a), 2(b), and 2(c) show the mechanograms of rat *muscle soleus* contraction at the application of an increasing stimulation signal with a frequency of 0–50 Hz for a duration of 5 s for an intact group of rats in Figure 2(a), groups of alcoholized rats in Figure 2(b), and rats receiving C₆₀FAS (1 mg kg^{-1}) along with alcohol in Figure 2(c).

Analysis of smooth contractile reactions (smooth tetanus) provides insight into the disruption of force generation by individual motor units [19]. The use of stimulation pools increasing in frequency made it possible to determine the time of establishment of smooth tetanic muscle contraction (t_1) in the experimental groups of animals (Figures 2 and 3(a)). In the intact group, the level of this index was $3.0 \pm 0.2 \text{ s}$ for the *muscle soleus* (Figure 3(a)). Three-month alcoholization did not significantly change the time to establish smooth tetanic contraction of *muscle soleus* (Figure 3(a)). At the same time, alcoholization during 6 and 9 months increased its level up to 3.2 ± 0.2 and $3.5 \pm 0.2 \text{ s}$, respectively. The application of C₆₀FAS reduced this time almost to the value in the intact group.

The time it takes for the muscle contraction force to decrease to zero after the cessation of stimulation (t_2) (Figures 2 and 3(b)) is an important indicator of muscle stiffness increase, which is associated with the formation of connective tissue components in the muscle [32]. The increase in stiffness opposes the contraction of the muscle fiber and its physiological transverse thickening during contraction, thereby prolonging the time required for the muscle to return to its initial state. Note, however, that this index depends not only on the contractile components but

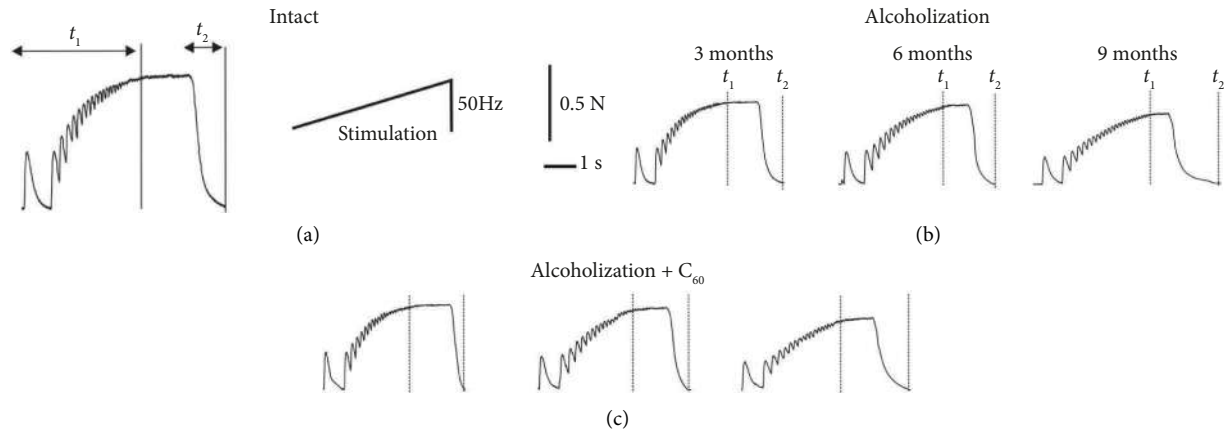


FIGURE 2: Force of *muscle soleus* contraction in alcoholized rats during modulated stimulation with increasing frequency (0–50 Hz) lasting 5 s: intact, alcoholization and alcoholization + C_{60} —an intact group of rats (a), groups of alcoholized rats (b) and rats receiving C_{60} FAS (1 mg kg^{-1}) along with alcohol (c), respectively; 3-, 6-, and 9-month-alcoholization for 3, 6 and 9 months, respectively; t_1 —time to establish smooth tetanic muscle contraction; t_2 —time for contraction force to decay to zero after stimulation cessation.

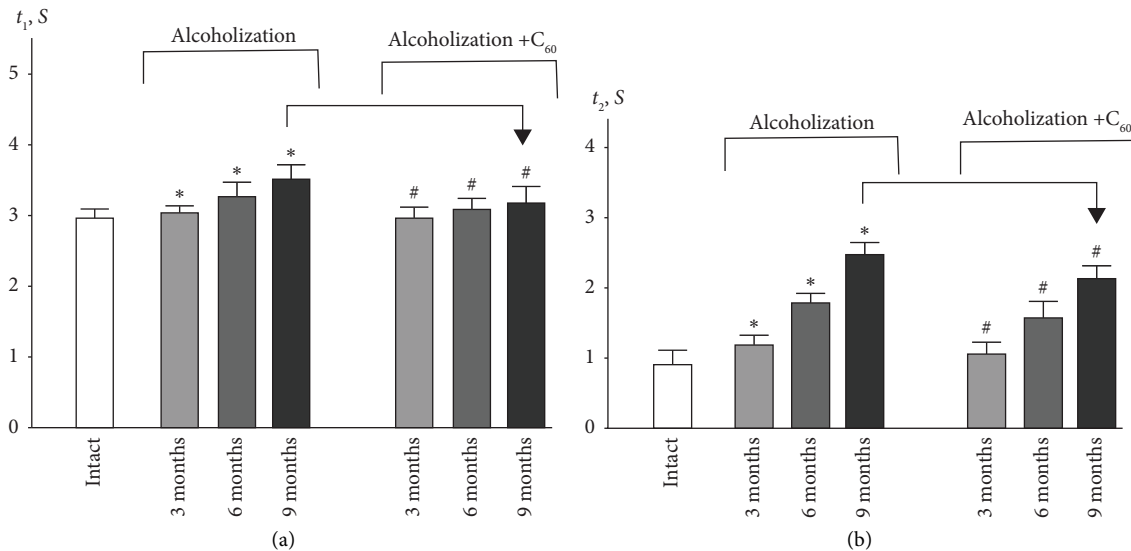


FIGURE 3: Mechanokinetic parameters of *muscle soleus* contraction of alcoholized rats during application of modulated stimulation of increasing frequency (0–50 Hz) with the duration of 5 s: t_1 —time to establish smooth tetanic muscle contraction (a); t_2 —time for muscle contraction force to decrease to zero after cessation of stimulation (b); intact, alcoholization, and alcoholization + C_{60} —an intact group of rats, groups of alcoholized rats, and rats receiving C_{60} FAS (1 mg kg^{-1}) along with alcohol, respectively; 3-, 6-, and 9-month-alcoholization for 3, 6 and 9 months, respectively; * $p < 0.05$ relative to the intact group; # $p < 0.05$ relative to the alcoholization group. Arrows indicate the most significant differences between the alcoholization and alcoholization + C_{60} groups.

also on the nature of the interaction between them and is therefore a marker of the development of myopathic disorders of muscle kinetics [1, 5].

Figure 3(b) shows that in the intact group, the time for the force to decrease to zero in the *muscle soleus* was 0.9 ± 0.1 s. Alcoholization led to a significant increase in this parameter, particularly at 6 and 9 months, reaching 1.8 ± 0.1 and 2.5 ± 0.1 s, respectively, indicating notable degenerative changes in the *muscle soleus*. The application of C_{60} FAS reduced this index nearly to the intact group level after 3 months of alcoholization and to 1.5 ± 0.1 and 2.1 ± 0.1 s at 6

and 9 months of alcoholization, respectively. The most positive effect of C_{60} FAS application was observed at 9 months, with $17 \pm 1\%$ improvement compared to the alcoholization group.

One of the effective approaches to identify the level of pathology in the muscle system is analyzing the development of fatigue processes during nonrelaxation stimulation of the muscle [18]. These processes play an important role in ensuring the precise positioning of movements of the musculoskeletal system. In this context, several biomechanical parameters were investigated as markers of muscle fatigue development [18, 19].

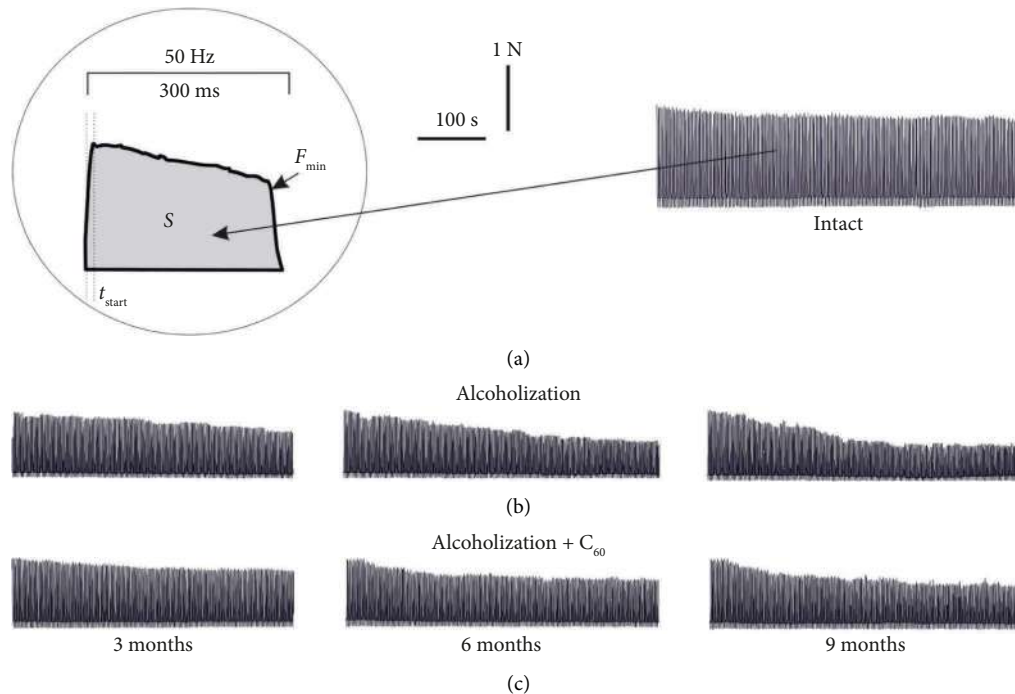


FIGURE 4: Mechanograms of 500 consecutive *muscle soleus* contractions of alcoholized rats during application of nonrelaxation pools of 50-Hz frequency lasting 3 s each: intact, alcoholization, and alcoholization + C_{60} —an intact group of rats (a), groups of alcoholized rats (b), and rats receiving C_{60} FAS (1 mg kg^{-1}) along with alcohol (c), respectively; 3-, 6-, and 9-month-alcoholization for 3, 6, and 9 months, respectively; $F_{\min}$: minimum force during a single contractile act; t_{start} : time to reach peak force response; S: integrated muscle power.

Figures 4(a), 4(b), and 4(c) show mechanograms of 500 consecutive contractions of *muscle soleus* in alcoholized rats when relaxation-free pools of 50-Hz frequency for a duration of 3 s each were applied for an intact group of rats Figure 4(a), groups of alcoholized rats Figure 4(b), and rats receiving C_{60} FAS Figure 4(c).

Analysis of the integrated muscle power (S) (Figures 4 and 5(a)) allows us to evaluate the formation of muscle activity in the “force-external load” system, which is essentially a physiological analog of the performance of the muscle system [19]. In the group of intact animals, this index decreased from 100% at the 1st contraction to $94 \pm 2\%$ at the 500th contraction of *muscle soleus* (Figure 5(a)). Testing of the *muscle soleus* revealed a significant decrease in the integrated muscle power at 6 and 9 months of alcoholization: $22 \pm 1\%$ and $30 \pm 1\%$ (1st contraction) and $30 \pm 1\%$ and $44 \pm 2\%$ (500th contraction), respectively, relative to the intact group. Application of C_{60} FAS increased this index, and at 6 and 9 months of alcoholization, its difference was $22 \pm 1\%$ and $37 \pm 1\%$ (500th contraction), respectively, relative to the alcoholization group (Figure 5(a)).

For comparison: In the case of the *muscle gastrocnemius* [19], the most positive effect of C_{60} FAS was $26 \pm 2\%$ following 9 months of alcoholization of animals.

The change in the level of minimal contraction force ($F_{\min}$) (Figures 4 and 5(b)) is the most sensitive indicator of muscle dysfunction caused by pathological processes [18]. Thus, this index for *muscle soleus* decreased by $25 \pm 1\%$ and $38 \pm 2\%$, $36 \pm 2\%$ and $50 \pm 2\%$, $51 \pm 2\%$ and $62 \pm 3\%$ at the 1st

and 500th contractions at 3, 6, and 9 months of alcoholization, respectively, relative to the intact group (Figure 5(b)). The application of C_{60} FAS increased this parameter by $11 \pm 1\%$ (1st contraction) and $23 \pm 1\%$ (500th contraction) at 3 and 6 months of alcoholization, respectively. At 9 months of alcoholization, the increase was $11 \pm 1\%$ (1st contraction) and $37 \pm 2\%$ (500th contraction) relative to the alcoholization group.

For comparison: In the case of the *muscle gastrocnemius* [19], the most positive effect of C_{60} FAS was $33 \pm 3\%$ following 9 months of alcoholization of animals.

The increasing time to reach the maximum force response (t_{start}) (Figures 4 and 5(c)) is also an important marker of dysfunctions in the muscle’s excitation-contraction system, as it indicates the possibility of blocking the accurate positioning of the joint during movement. Thus, this index increased by $38 \pm 2\%$ and $30 \pm 2\%$, $43 \pm 2\%$ and $45 \pm 2\%$, $52 \pm 3\%$ and $55 \pm 3\%$ for the 1st and 500th *muscle soleus* contractions at 3, 6, and 9 months of alcoholization, respectively, compared to the intact group (Figure 5(c)). The positive effect of C_{60} FAS was $14 \pm 1\%$ and $22 \pm 1\%$, $12 \pm 1\%$ and $20 \pm 1\%$, $15 \pm 1\%$ and $25 \pm 1\%$, respectively, relative to the alcoholization group.

In summary, the most significant biomechanical effects of C_{60} FAS (over 25%) occur at long-term alcoholization in animals (9 months) under the development of high-intensity fatigue in the *muscle soleus*, which agrees with the results for the *muscle gastrocnemius* [19].

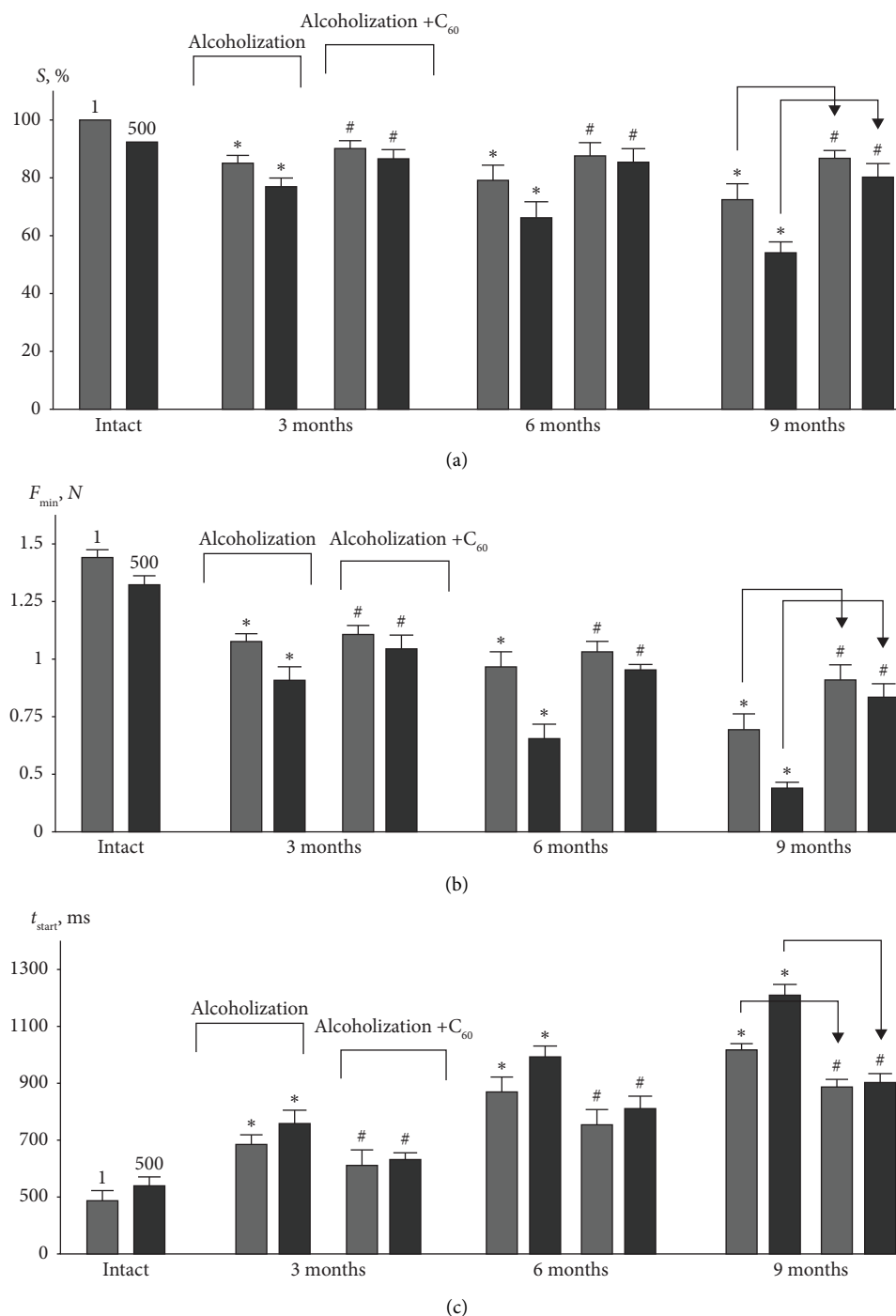


FIGURE 5: Mechanokinetic parameters of *muscle soleus* contraction in alcoholized rats during application of nonrelaxation pools of 50-Hz frequency with the duration of 3 s each: S: integrated muscle power calculated as the area under the force curve (taken as 100% at the first contraction of the muscle in the intact group) (a); $F_{\min}$: minimum force during a single contractile act (b); t_{start} : time to reach peak force response (c); 1 and 500: 1st and 500th muscle contraction, respectively; intact, alcoholization and alcoholization + C₆₀—an intact group of rats, groups of alcoholized rats, and rats receiving C₆₀FAS (1 mg/kg⁻¹) along with alcohol, respectively; 3-, 6-, and 9-month-alcoholization for 3, 6, and 9 months, respectively; * $p < 0.05$ relative to the intact group; # $p < 0.05$ relative to the alcoholization group. Arrows indicate the most significant differences between the alcoholization and alcoholization + C₆₀ groups.

3.3. Biochemical Analysis of Muscle Tissues. The BAC in alcoholized rats ranged from $140 \pm 5 \text{ mg dL}^{-1}$ (3 months of alcohol consumption) to $261 \pm 6 \text{ mg dL}^{-1}$ (9 months of alcohol

consumption). These results agree well with previously reported data [23]. The administration of C₆₀FAS alongside ethanol did not significantly alter the BAC values [18–20].

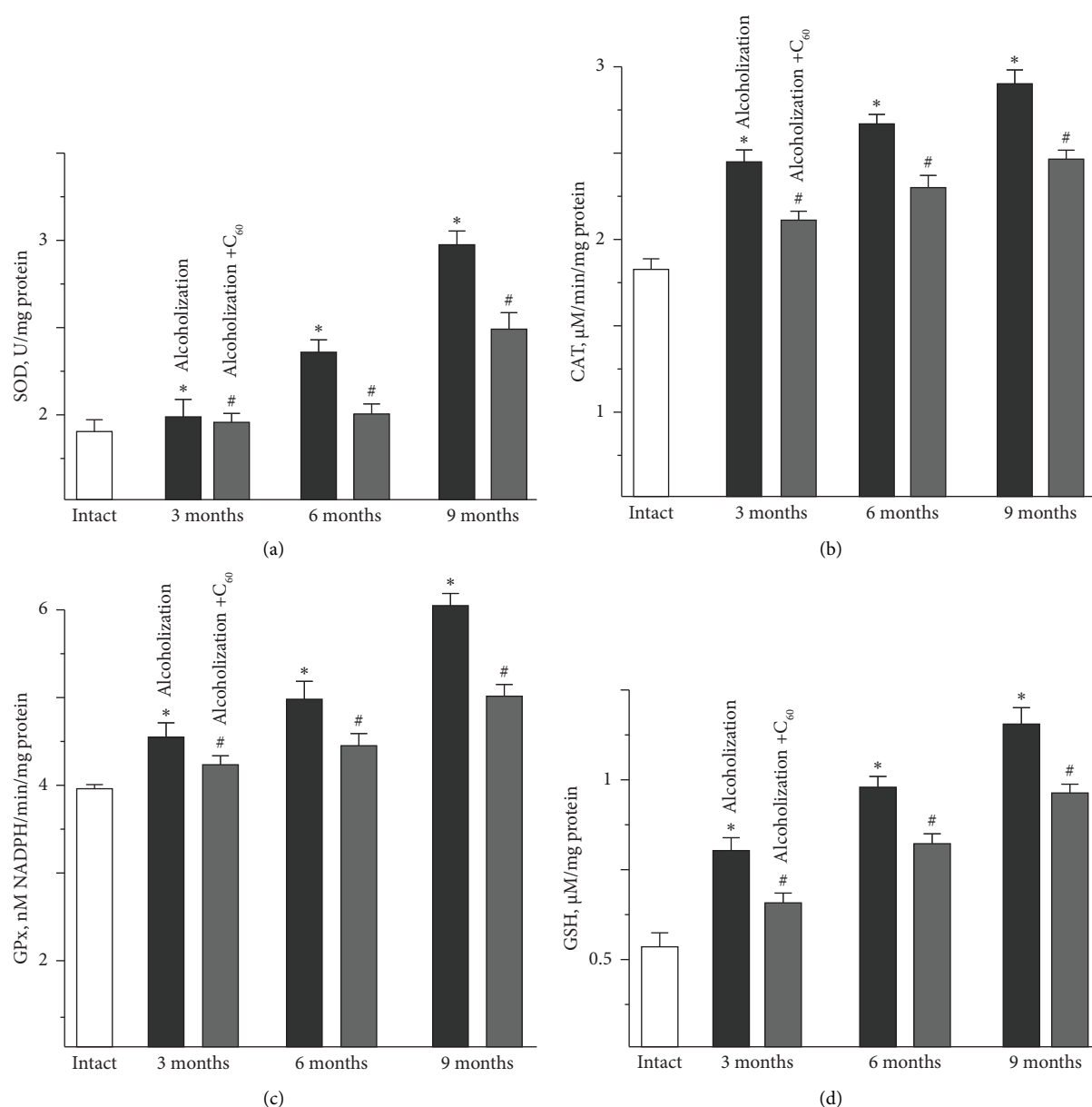


FIGURE 6: Indicators of pro- and antioxidant balance (SOD (a), CAT (b), GP_x (c), and GSH (d)) in the *muscle soleus* tissues of alcoholized rats after application of stimulation: intact, alcoholization, and alcoholization + C₆₀—an intact group of rats, groups of alcoholized rats, and rats receiving C₆₀FAS (1 mg kg⁻¹) along with alcohol, respectively; 3-, 6-, and 9-month-alcoholization for 3, 6, and 9 months, respectively; **p* < 0.05 relative to the intact group; #*p* < 0.05 relative to the alcoholization group. Arrows indicate the most significant differences between the alcoholization and alcoholization + C₆₀ groups.

Antioxidant defense enzymes, particularly SOD and CAT, play a critical role in regulating ROS and peroxide processes [33]. Research [34] has demonstrated that under conditions of physical exercise, SOD activity in the skeletal muscles of animals increases predominantly in oxidative muscles without significant changes in mRNA expression. In our experiment, SOD activity in the *muscle soleus* increased by 11 ± 1%, 18 ± 1%, and 36 ± 2% at 3, 6, and 9 months of alcoholization, respectively, compared to the intact group (Figure 6(a)). The most pronounced positive effect of C₆₀FAS was observed at 9 months of alcoholization, with an increase of 25 ± 1% relative to the alcoholization group.

For comparison: In the case of *muscle gastrocnemius* [19], the most positive effect of C₆₀FAS was 44 ± 2% following 9 months of alcoholization of animals.

The increase in CAT activity in the *muscle soleus* was as follows: 21 ± 1%, 27 ± 1%, and 32 ± 2% at 3, 6, and 9 months of alcoholization, respectively, relative to the intact group (Figure 6(b)). The most pronounced positive effect of C₆₀FAS was observed at 9 months of alcoholization with a 20 ± 1% increase relative to the alcoholization group.

For comparison: In the case of the *muscle gastrocnemius* [19], the most positive effect of C₆₀FAS was 48 ± 2% following 9 months of alcoholization of animals.

Despite a significant body of research, data on CAT activity under conditions of intensive physical exercise and the development of pathologies in skeletal muscle remain contradictory. Thus, it was found [34] that chronic exercise does not alter CAT activity in slow muscle but reduces it in fast muscle. Research [35] reported a decrease in CAT activity in both oxidative and glycolytic types of muscle fibers. Finally, the authors [36] recorded an increase in CAT activity during exercise in both muscle types.

Along with antiradical enzymes, the GSH link is an active component of the antioxidant defense of the organism [33, 37–39]. The increase in GP_x activity in the *muscle soleus* was $11 \pm 1\%$, $18 \pm 1\%$, and $27 \pm 1\%$ at 3, 6, and 9 months of alcoholization, respectively, relative to the intact group (Figure 6(c)). The most positive effect of C₆₀FAS was $21 \pm 1\%$ relative to the alcoholization group for 9 months of alcoholization.

For comparison: In the case of the *muscle gastrocnemius* [19], the most positive effect of C₆₀FAS was $39 \pm 2\%$ following 9 months of alcoholization of animals.

GSH content in the *muscle soleus* increased by $25 \pm 1\%$, $33 \pm 2\%$, and $99 \pm 4\%$ at 3, 6, and 9 months of alcoholization, respectively, relative to the intact group (Figure 6(d)). The most positive effect of C₆₀FAS was $23 \pm 1\%$ at 9-month alcoholization relative to the alcoholization group.

For comparison: In the case of the *muscle gastrocnemius* [19], the most positive effect of C₆₀FAS was $42 \pm 2\%$ following 9 months of alcoholization of animals.

Thus, the most significant biochemical effects of C₆₀FAS (exceeding 20%) are observed during long-term (9 months) alcohol administration in animals, which agrees with the results for the *muscle gastrocnemius* [19].

In summary, the application of C₆₀ fullerenes contributes to the reduction of oxidative processes in both the *soleus* and *gastrocnemius* muscles of rats. This can be attributed to the maintenance of a balance between the antioxidant defense system and pro-oxidants, which mitigates the negative effect of ROS on cellular and subcellular structures during the development of muscle fatigue in alcohol-exposed rats [27].

Possible molecular mechanisms describing the obtained data require further detailed study, since, for example, the use of such known antioxidants as carotenoids, vitamins, polyphenols, and flavonoids in treating chronic alcoholism demonstrates contradictory results [13]. For today, the following mechanism of C₆₀ fullerene action can be considered. In alcoholic myopathy, ROS are formed in dysfunctional mitochondria [10], which leads to the destruction of cell membranes and functional disorders of their enzyme systems. So, a decrease in the activity of Na⁺/K⁺-ATPase inhibits the specific increase in Na⁺ conductivity that occurs in response to an adequate stimulus and thus prevents the development of excitation in myocytes. This can lead to a temporary imbalance in the time of establishment of smooth tetanus, which, in turn, increases the time required for the muscle to perform a force exercise. On the other hand, it is known that over 90% of alcohol in the body is metabolized through oxidative and nonoxidative pathways, resulting in the formation of highly reactive compounds that

also actively generate ROS [40]. C₆₀ fullerenes as powerful exogenous nanoantioxidants [16, 26, 41] with mitochondrial targeting [15] effectively inactivate ROS and thus reduce dysfunctions in *muscle soleus* of rats with chronic alcohol intoxication.

It has shown [42] that C₆₀ fullerenes are capable of effectively adsorbing various aromatic molecules on their surface. It can be assumed that nanoaggregates of C₆₀ fullerenes present in ethanol are capable of binding its molecules and, thus, entering the body, reduce the negative impact of alcohol on the muscular system functioning, which requires theoretical verification of this hypothesis.

4. Conclusions

The obtained biomechanical and biochemical data on the functioning of rat *muscle soleus* indicate that after 3, 6, and 9 months of alcoholization, the experimental animals develop significant dysfunctions in the skeletal musculature. A significant therapeutic effect of C₆₀ fullerenes (at an oral dose of 1 mg kg^{-1} along with alcohol throughout the experiment) was observed in correcting the contractile activity of the alcoholized *muscle soleus*, as evidenced by the studied biomechanical and biochemical parameters (on average over 20%). This effect is attributed to the modification of ROS-dependent mechanisms, which play a critical role in alcoholic myopathy. These findings highlight the potential of C₆₀ fullerenes for correcting pathological states of the skeletal muscles induced by alcohol intoxication.

Data Availability Statement

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request. Apart from the data sets used, no Supporting Information is available.

Ethics Statement

The study protocol was approved by the Biomedical Ethics Committee of the ESC “Institute of Biology and Medicine” of Taras Shevchenko National University of Kyiv (protocol No. 2 dated September 2, 2022) and performed following Article 26 of the Law of Ukraine “On the Protection of Animals from Cruelty” (No. 3447-IV, 21.02.2006), as well as European Union Directive of 22 September 2010 (2010/63/EU) for the protection of animals used for scientific purposes.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Olexandr Motuziuk and Dmytro Nozdrenko: methodology, biomechanical analysis, analysis of the data, and preparation of the manuscript. Svitlana Prylutska, Kateryna Bogutska, and Olena Dmytrotso: biochemical analysis. Yurii Osyp and Peter Scharff: preparation and characterization of the

samples and analysis of the data. Yuriy Prylutsky: conceptualization, methodology, coordination of the research work, analysis of the data, preparation of the manuscript, writing – original draft preparation, and writing and review–editing. Uwe Ritter: coordination of the research work, preparation of the manuscript, and writing and review–editing.

Funding

We acknowledge support for the publication costs by the Open Access Publication Fund of the Technical University of Ilmenau, Germany.

Acknowledgments

We acknowledge support for the publication costs by the Open Access Publication Fund of the Technical University of Ilmenau, Germany.

References

- [1] V. R. Preedy, K. Ohlendieck, J. Adachi, et al., “The Importance of Alcohol-Induced Muscle Disease,” *Journal of Muscle Research and Cell Motility* 24, no. 1 (2003): 55–63, <https://doi.org/10.1023/a:1024842817060>.
- [2] R. Preedy and T. J. Peters, “The Effect of Chronic Ethanol Ingestion on Protein Metabolism in Type-I- and Type-II-Fibre-Rich Skeletal Muscles of the Rat,” *Biochemical Journal* 254, no. 3 (1988): 631–639, <https://doi.org/10.1042/bj2540631>.
- [3] M. E. Reilly, G. McKoy, D. Mantle, T. J. Peters, G. Goldspink, and V. R. Preedy, “Protein and mRNA Levels of the Myosin Heavy Chain Isoforms Ibeta, Ila, IIX and IIb in Type I and Type II Fibre-Predominant Rat Skeletal Muscles in Response to Chronic Alcohol Feeding,” *Journal of Muscle Research & Cell Motility* 21, no. 8 (2000): 763–773, <https://doi.org/10.1023/a:1010336624154>.
- [4] S. Thapaliya, A. Runkana, M. R. McMullen, et al., “Alcohol-Induced Autophagy Contributes to Loss in Skeletal Muscle Mass,” *Autophagy* 10, no. 4 (2014): 677–690, <https://doi.org/10.4161/auto.27918>.
- [5] K. T. Crowell, L. J. Laufenberg, and C. H. Lang, “Chronic Alcohol Consumption, But Not Acute Intoxication, Decreases *In Vitro* Skeletal Muscle Contractile Function,” *Alcoholism: Clinical and Experimental Research* 43, no. 10 (2019): 2090–2099, <https://doi.org/10.1111/acer.14179>.
- [6] B. S. Shenkman, O. E. Zinovyeva, S. P. Belova, et al., “Cellular and Molecular Signatures of Alcohol-Induced Myopathy in Women,” *American Journal of Physiology, Endocrinology and Metabolism* 316, no. 5 (2019): E967–E976, <https://doi.org/10.1152/ajpendo.00513.2018>.
- [7] R. G. Taylor, R. T. Abresch, J. S. Lieberman, R. B. Sharman, R. K. Enrikin, and W. M. Fowler Jr., “Fast and Slow Skeletal Muscles: Effect of Ethanol on Contractility of Muscles From Mice,” *Archives of Physical Medicine and Rehabilitation* 73, no. 4 (1992): 355–358, [https://doi.org/10.1016/0003-9993\(92\)90009-1](https://doi.org/10.1016/0003-9993(92)90009-1).
- [8] M. J. Barnes, T. Mundel, and S. R. Stannard, “Post-Exercise Alcohol Ingestion Exacerbates Eccentric-Exercise Induced Losses in Performance,” *European Journal of Applied Physiology* 108, no. 5 (2010): 1009–1014, <https://doi.org/10.1007/s00421-009-1311-3>.
- [9] J. S. Otis and D. M. Guidot, “Procysteine Stimulates Expression of Key Anabolic Factors and Reduces Plantaris Atrophy in Alcohol-Fed Rats,” *Alcoholism: Clinical and Experimental Research* 33, no. 8 (2009): 1450–1459, <https://doi.org/10.1111/j.1530-0277.2009.00975.x>.
- [10] J. Adachi, M. Asano, Y. Ueno, et al., “Alcoholic Muscle Disease and Biomembrane Perturbations (Review),” *The Journal of Nutritional Biochemistry* 14, no. 11 (2003): 616–625, [https://doi.org/10.1016/s0955-2863\(03\)00114-1](https://doi.org/10.1016/s0955-2863(03)00114-1).
- [11] O. Pansarasa, G. Felzani, J. Vecchiet, and F. Marzatico, “Antioxidant Pathways in Human Aged Skeletal Muscle: Relationship With the Distribution of Type II Fibers,” *Experimental Gerontology* 37, no. 8-9 (2002): 1069–1075, [https://doi.org/10.1016/s0531-5565\(02\)00085-2](https://doi.org/10.1016/s0531-5565(02)00085-2).
- [12] R. Koo-Ng, G. Falkous, M. Reilly, T. J. Peters, D. Mantle, and V. R. Preedy, “Carbonyl Levels in Type I and II Fiber-Rich Muscles and Their Response to Chronic Ethanol Feeding *In Vivo* and Hydroxyl and Superoxide Radicals *In Vitro*,” *Alcoholism: Clinical and Experimental Research* 24, no. 12 (2000): 1862–1868, <https://doi.org/10.1097/0000374-200012000-00016>.
- [13] C. S. Silva, G. V. Portari, and H. Vannucchi, “Antioxidant Treatment and Alcoholism,” in *Molecular Aspects of Alcohol and Nutrition* (Elsevier, 2016), 119–131, <https://doi.org/10.1016/B978-0-12-800773-0.00010-0>.
- [14] T. Halenova, N. Raksha, O. Savchuk, et al., “Evaluation of the Biocompatibility of Water-Soluble Pristine C₆₀ Fullerenes in Rabbit,” *BioNanoScience* 10, no. 3 (2020): 721–730, <https://doi.org/10.1007/s12668-020-00762-w>.
- [15] S. Foley, C. Crowley, M. Smahli, et al., “Cellular Localisation of a Water-Soluble Fullerene Derivative,” *Biochemical and Biophysical Research Communications* 294, no. 1 (2002): 116–119, [https://doi.org/10.1016/S0006-291X\(02\)00445-X](https://doi.org/10.1016/S0006-291X(02)00445-X).
- [16] C. A. Ferreira, D. Ni, Z. T. Rosenkrans, and W. Cai, “Scavenging of Reactive Oxygen and Nitrogen Species With Nanomaterials,” *Nano Research* 11, no. 10 (2018): 4955–4984, <https://doi.org/10.1007/s12274-018-2092-y>.
- [17] F. Moussa, “Fullerene and Derivatives for Biomedical Applications,” in *Book: Nanobiomaterials* (2018), 113–136, <https://doi.org/10.1016/B978-0-08-100716-7.00005-2>.
- [18] O. Motuziuk, D. Nozdrenko, S. Prylutska, et al., “C₆₀ Fullerene Reduces the Level of Fluctuations in the Force Response of *Muscle Gastrocnemius* in Chronically Alcoholized Rats,” *Applied Nanoscience* 13, no. 10 (2023): 7057–7067, <https://doi.org/10.1007/s13204-023-02874-7>.
- [19] O. Motuziuk, D. Nozdrenko, S. Prylutska, et al., “The Effect of C₆₀ Fullerene on the Mechanokinetics of *Muscle Gastrocnemius* Contraction in Chronically Alcoholized Rats,” *Heliyon* 9, no. 8 (2023): e18745, <https://doi.org/10.1016/j.heliyon.2023.e18745>.
- [20] O. Motuziuk, D. Nozdrenko, S. Prylutska, et al., “C₆₀ Fullerene Reduces the Level of Liver Damage in Chronic Alcohol Intoxication of Rats,” *Molecules* 29, no. 13 (2024): 2951, <https://doi.org/10.3390/molecules29132951>.
- [21] L. M. Skivka, S. V. Prylutska, M. P. Rudyk, et al., “C₆₀ Fullerene and its Nanocomplexes With Anticancer Drugs Modulate Circulating Phagocyte Functions and Dramatically Increase ROS Generation in Transformed Monocytes,” *Cancer Nanotechnology* 9, no. 1 (2018): 8, <https://doi.org/10.1186/s12645-017-0034-0>.
- [22] D. Nozdrenko, O. Motuziuk, S. Prylutska, et al., “C₆₀ Fullerene Protective Effect Against the Rat *Muscle Soleus* Trauma,” *Heliyon* 10, no. 11 (2024): e32677, <https://doi.org/10.1016/j.heliyon.2024.e32677>.

- [23] K. Song, R. A. Coleman, X. Zhu, et al., "Chronic Ethanol Consumption by Mice Results in Activated Splenic T Cells," *Journal of Leukocyte Biology* 72, no. 6 (2002): 1109–1116, <https://doi.org/10.1189/jlb.72.6.1109>.
- [24] M. A. Collins and E. J. Neafsey, "Alcohol, Excitotoxicity, and Adult Brain Damage: An Experimentally Unproven Chain-of-Events," *Frontiers in Molecular Neuroscience* 9 (2016): 8, <https://doi.org/10.3389/fnmol.2016.00008>.
- [25] N. B. D'Souza El-Guindy, E. J. Kovacs, P. De Witte, et al., "Laboratory Models Available to Study Alcohol-Induced Organ Damage and Immune Variations: Choosing the Appropriate Model," *Alcoholism: Clinical and Experimental Research* 34, no. 9 (2010): 1489–1511, <https://doi.org/10.1111/j.1530-0277.2010.01234.x>.
- [26] N. Gharbi, M. Pressac, M. Hadchouel, H. Szwarc, S. R. Wilson, and F. Moussa, "C₆₀ Fullerene Is a Powerful Antioxidant In Vivo With No Acute or Subacute Toxicity," *Nano Letters* 5, no. 12 (2005): 2578–2585, <https://doi.org/10.1021/nl051866b>.
- [27] Yu. Prylutsky, D. Nozdrenko, O. Motuziuk, et al., "C₆₀ Fullerene Improves the Contractile Activity of the Injured Rat Muscle *Gastrocnemius*," *Nanotechnology* 36, no. 12 (2025): 125101, <https://doi.org/10.1088/1361-6528/ada29a>.
- [28] P. Brancaccio, G. Lippi, and N. Maffulli, "Biochemical Markers of Muscular Damage," *Clinical Chemistry and Laboratory Medicine* 48, no. 6 (2010): 757–767, <https://doi.org/10.1515/CCLM.2010.179>.
- [29] C. Egbuna, V. K. Parmar, J. Jeevanandam, et al., "Toxicity of Nanoparticles in Biomedical Application: Nanotoxicology," *Journal of Toxicology* 2021 (2021): 1–21, <https://doi.org/10.1155/2021/9954443>.
- [30] R. Abbasi, G. Shineh, M. Mobaraki, S. Doughty, and L. Tayebi, "Structural Parameters of Nanoparticles Affecting Their Toxicity for Biomedical Applications: A Review," *Journal of Nanoparticle Research* 25, no. 3 (2023): 43, <https://doi.org/10.1007/s11051-023-05690-w>.
- [31] Yu. Prilutski, S. Durov, L. Bulavin, et al., "Study of the Structure of Colloidal Particles of Fullerenes in Water Solution," *Molecular Crystals and Liquid Crystals* 24 (1998): 65–70, <https://doi.org/10.1080/10587259808047135>.
- [32] A. J. Wade, M. M. Marbut, and J. M. Round, "Muscle Fibre Type and Aetiology of Obesity," *The Lancet* 335, no. 8693 (1990): 805–808, [https://doi.org/10.1016/0140-6736\(90\)90933-v](https://doi.org/10.1016/0140-6736(90)90933-v).
- [33] C. K. Sen, "Antioxidant and Redox Regulation of Cellular Signaling: Introduction," *Medicine, Science in Sports and Exercise* 33, no. 3 (2001): 368–370, <https://doi.org/10.1097/00005768-200103000-00005>.
- [34] K. Suzuki, H. Ohno, S. Oh-ishi, et al., "Superoxide Dismutases in Exercise and Disease," in *Handbook of Oxidants and Antioxidants and Exercise*, ed. C. K. Sen, L. Packer, and O. Hanninen (Amsterdam: Elsevier, 2000), 243–295, <https://doi.org/10.1016/B978-044482650-3/50011-0>.
- [35] O. Gonchar, "Muscle Fiber Specific Antioxidative System Adaptation to Swim Training in Rats: Influence of Intermittent Hypoxia," *Journal of Sports Science and Medicine* 4, no. 2 (2005): 160–169.
- [36] J. Hollander, R. Fiebig, M. Gore, et al., "Superoxide Dismutase Gene Expression in Skeletal Muscle: Fiber-Specific Adaptation to Endurance Training," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 277, no. 3 (1999): R856–R862, <https://doi.org/10.1152/ajpregu.1999.277.3.R856>.
- [37] M. Vogt, A. Puntchart, J. Geiser, C. Zuleger, R. Billeter, and H. Hoppeler, "Molecular Adaptations in Human Skeletal Muscle to Endurance Training Under Simulated Hypoxic Conditions," *Journal of Applied Physiology* 91, no. 1 (2001): 173–182, <https://doi.org/10.1152/jappl.2001.91.1.173>.
- [38] G. Wu, J. R. Lupton, N. D. Turner, Y.-Z. Fang, and S. Yang, "Glutathione Metabolism and Its Implications for Health," *The Journal of Nutrition* 134, no. 3 (2004): 489–492, <https://doi.org/10.1093/jn/134.3.489>.
- [39] L. L. Ji, "Modulation of Skeletal Muscle Antioxidant Defense by Exercise: Role of Redox Signaling," *Free Radical Biology and Medicine* 44, no. 2 (2008): 142–152, <https://doi.org/10.1016/j.freeradbiomed.2007.02.031>.
- [40] M. A. Schukit, "Ethanol and Methanol," in *Goodman & Gillman's Pharmacological Basis of Therapeutics*, ed. L. L. Brunton (New Delhi: McGraw Hill, 2011), 629–647.
- [41] S. V. Eswaran, "Water Soluble Nanocarbon Materials: A Panacea for All?" *Current Science* 114, no. 09 (2018): 1846–1850, <https://doi.org/10.18520/cs/v114/i09/1846-1850>.
- [42] M. P. Evstigneev, A. S. Buchelnikov, D. P. Voronin, et al., "Complexation of C₆₀ Fullerene With Aromatic Drugs," *ChemPhysChem* 14, no. 3 (2013): 568–578, <https://doi.org/10.1002/cphc.201200938>.



OPEN The effect of C₆₀ fullerene on the *musculus gastrocnemius* contraction in chronically alcohol-exposed rats and the potential mechanism of its action

Olexandr Motuziuk^{1,2}, Dmytro Nozdrenko², Svitlana Prylutska³, Nina Tverdokhleba⁴, Mykola Petrovsky², Sergiy Vyzhva², Yuriy Prylutsky², Peter Scharff⁵ & Uwe Ritter⁵✉

Over 90% of consumed alcohol is metabolized through oxidative and non-oxidative pathways, producing highly reactive compounds capable of generating reactive oxygen species (ROS). ROS induce increased oxidative stress and lipid peroxidation, thereby disrupting the structural integrity of myocytes and the functions of skeletal muscles in general. It is hypothesized that biocompatible and bioavailable C₆₀ fullerenes, as potent antioxidants, can effectively absorb ROS, normalizing the functional state of the muscular system during the chronic alcoholic myopathy (CAM). Here, for the first time, the effect of C₆₀ fullerenes (oral daily dose of 1 mg/kg) administered together with alcohol (40% ethanol in drinking water) on the contractile activity of the *musculus gastrocnemius* in male Wistar rats (age 1 month, weight 170 ± 10 g; n = 36) during the development of CAM over 3, 6, and 9 months was analyzed using tensometry. Biochemical analysis was used to evaluate pro- and antioxidant balance indicators in the blood of alcohol-exposed animals under the influence of C₆₀ fullerenes. Finally, the potential mechanism of action of C₆₀ fullerenes under chronic alcohol intoxication in rats was analyzed using a computer simulation technique. The data obtained indicate an improvement in the studied biomechanical markers of contraction in alcohol-exposed *musculus gastrocnemius* with a range of 16–50 ± 3%. Additionally, significant biochemical changes were observed in the pro- and antioxidant balance in the blood of experimental rats, showing an improvement of 22–39 ± 2% due to the action of C₆₀ fullerenes, compared to the alcohol-exposed group. It is demonstrated that C₆₀ fullerene nanoparticles can bind ethanol molecules, thereby reducing the negative effects of alcohol on the functioning of the muscular system.

Keywords *Musculus gastrocnemius*, Chronic alcohol intoxication, C₆₀ fullerene, Biomechanical parameters of muscle contraction, Indicators of pro- and antioxidant balance in the blood, Computer simulation

Chronic alcohol use disorder (AUD)¹ is a disease characterized by a syndrome of addictive alcohol dependence, which leads to specific somatic, myopathic, and neurological disorders in the body. According to the WHO, more than 3.3 million people worldwide die annually due to AUD^{2,3}. Notably, approximately 25% of all deaths associated with chronic AUD occur among young people aged 20–39 years⁴.

Alcoholic myopathy is recognized as a distinct pathological condition that arises from other manifestations of alcoholic disease, such as liver damage, myocardial injury, or alcoholic polyneuropathy⁵. Several clinical forms of alcoholic myopathy are identified as those developing against acute or chronic alcohol intoxication and are collectively referred to as “alcohol-induced muscle damage”⁶. The most prevalent form is chronic alcoholic myopathy (CAM), which occurs in 40–60% of individuals who abuse alcohol^{7,8}. Alcohol reduces the

¹Faculty of Biology and Forestry, Lesya Ukrainka Volyn National University, Lutsk 43025, Ukraine. ²ESC “Institute of Biology and Medicine”, Taras Shevchenko National University of Kyiv, Kyiv 01601, Ukraine. ³Faculty of Plant Protection, Biotechnology and Ecology, National University of Life and Environmental Science of Ukraine, Kyiv 03041, Ukraine. ⁴Institute for Materials Science and Max Bergmann Center for Biomaterials, TUD Dresden University of Technology, 01062 Dresden, Germany. ⁵Institute of Chemistry and Biotechnology, Technical University of Ilmenau, 98693 Ilmenau, Germany. ✉email: uwe.ritter@tu-ilmenau.de

differentiation potential of muscle stem cells and disrupts the regulation of the extracellular matrix, thereby impairing the muscle's regenerative capacity⁹. CAM encompasses a complex array of biochemical, physiological, and structural changes in skeletal muscle resulting from prolonged alcohol intoxication, leading to loss of muscle strength and mass¹⁰. Many symptoms, including acute muscle weakness, tonic spasms, high levels of tremor, and myoglobinuria with a high risk of acute renal failure¹¹ characterize CAM. Importantly, CAM is not accompanied by a significant increase in the level of the enzyme creatine phosphokinase in the blood, and electromyography does not reveal changes typical of primary muscle damage, such as reduced amplitude and duration of motor unit action potentials¹² which often complicates its clinical diagnosis. It has been shown that a morphological indication of CAM development is muscle fiber atrophy, with its severity proportional to the duration of alcohol consumption and weakly dependent on dosage^{13,14}. Progressive muscle fiber atrophy can lead to significant loss of muscle mass¹⁵.

The development of CAM is partially attributed to altered redox states and an imbalance of endogenous antioxidants in the body¹⁶. These impairments lead to a reduction in ATP content in skeletal muscle and a diminished redox ratio (NAD^+/NADH), accompanied by increased electron leakage, primarily from complex III (ubiquinone), which promotes the generation of mitochondrial reactive oxygen species (ROS) and reverse electron transport. Collectively, these processes contribute to mitochondrial dysfunction in skeletal muscle, suppressed protein synthesis, and enhanced autophagy¹⁷. Alcohol enhances the activity of cytochrome P450, which also facilitates the formation of ROS. Finally, ethanol metabolism in the liver produces highly reactive compounds, and their further metabolism is also linked to the generation of ROS¹⁸.

The effective inactivation of ROS in both in vitro and in vivo systems by biocompatible and bioavailable C_{60} fullerene nanoparticles presents promising opportunities for their extensive biomedical applications^{19,20}. It was shown that C_{60} fullerenes have more potent antioxidant properties compared to natural antioxidants vitamins C and E²¹ as well as the exogenous antioxidants N-acetylcysteine (NAC) and β -alanine²² which are often used in sports medicine. Notably, mitochondria are the primary organelles responsible for the production of ROS in damaged skeletal muscles. Because of this, C_{60} fullerenes with mitochondrial targeting²³ are powerful scavengers of ROS induced by muscle pathologies of various kinds^{24,25}.

Previously, on the models of alcohol-exposed rats²⁶ it was shown that simultaneous administration of alcohol (40% ethanol in drinking water) and C_{60} fullerenes (the optimal daily dose of 1 mg/kg) results in a higher level of *musculus gastrocnemius* force response correction compared to other methods of this drug administration (1 h before and 1 h after alcohol intake). In particular, this therapeutic approach reduces fluctuations in force response during maximum force effort (the so-called "tremor"). Moreover, C_{60} fullerenes, which were administered to rats together with alcohol, reduced the time occurrence of fatigue processes in *musculus gastrocnemius* during the long-term development of alcoholic myopathy and inhibited oxidative processes in muscle, thereby preventing its degradation²⁷.

Therefore, this study aimed to analyze in detail the effect of C_{60} fullerenes administered along with alcohol for 3, 6, and 9 months on the contractile activity of the *musculus gastrocnemius* in rats by evaluating (1) the most informative biomechanical markers of muscle dysfunction and biochemical indicators of pro- and antioxidant balance in the blood, as well as (2) the potential mechanism of C_{60} fullerene nanoparticles action using a computer simulation technique.

Results

Weight of experimental animals

In the control group, the average body weight of the rats increased from 170 ± 10 g at the beginning of the experiment to 210 ± 11 g, 240 ± 13 g, and 290 ± 15 g at the end of months 3, 6, and 9, respectively. In the alcohol-exposed group, body weight increased from 170 ± 10 g to 200 ± 10 g, 220 ± 12 g, and 250 ± 13 g over the same time points. Finally, in the alcohol + C_{60} -treated group, average body weight did not differ reliably from that of the alcohol-only group.

Biomechanical analysis of *musculus gastrocnemius* contraction in alcohol-exposed rats

Muscle weakness is the most prevalent clinical manifestation of CAM²⁸ characterized by a reduction in muscle contractile force and altered timing in achieving force parameters that correspond to the frequency of efferent stimulation observed in an intact muscle. To assess these myopathic impairments in the *musculus gastrocnemius*, we applied a stimulation signal at a frequency of 50 Hz for a duration of 5 s (Fig. 1).

Figure 2 shows that after 3 months of alcohol-exposed animals, the value of integrated muscle power, as a physiological analog of its performance²⁹ significantly decreased during all stimulation pools and at the 5th contraction amounted to $57 \pm 3\%$ of the control value. The use of C_{60} fullerene revealed an improvement of this parameter by $16 \pm 1\%$ compared to the alcohol-exposed group.

The integrated muscle power of rats after 6 months of AUD decreased to $29 \pm 1\%$ of the control at the 5th contraction. The use of C_{60} fullerene improved this indicator by $21 \pm 1\%$ compared to the alcohol-exposed group (Fig. 2).

Finally, AUD for 9 months significantly decreased the integrated muscle power, which amounted to $10 \pm 1\%$ of the control at the 5th contraction. The use of C_{60} fullerene increased this value by $23 \pm 1\%$ compared to the alcohol-exposed group (Fig. 2).

As can be seen from Fig. 3, the time of the beginning of the muscle force response increased from 150 ± 15 ms in the control to 300 ± 20 , 390 ± 15 , and 480 ± 22 ms at 3, 6, and 9 months of alcohol consumption, respectively. The use of C_{60} fullerenes reduced this parameter to 185 ± 8 , 210 ± 14 , and 240 ± 16 ms, respectively, which was $38 \pm 2\%$, $46 \pm 3\%$, and $50 \pm 4\%$ compared to the alcohol-exposed group.

As can be seen from Fig. 4, the time for the muscle force response to reach its initial level increased from 500 ± 17 ms in control to 690 ± 21 , 900 ± 27 and 1200 ± 29 ms at 3.6 and 9 months of AUD, respectively. With

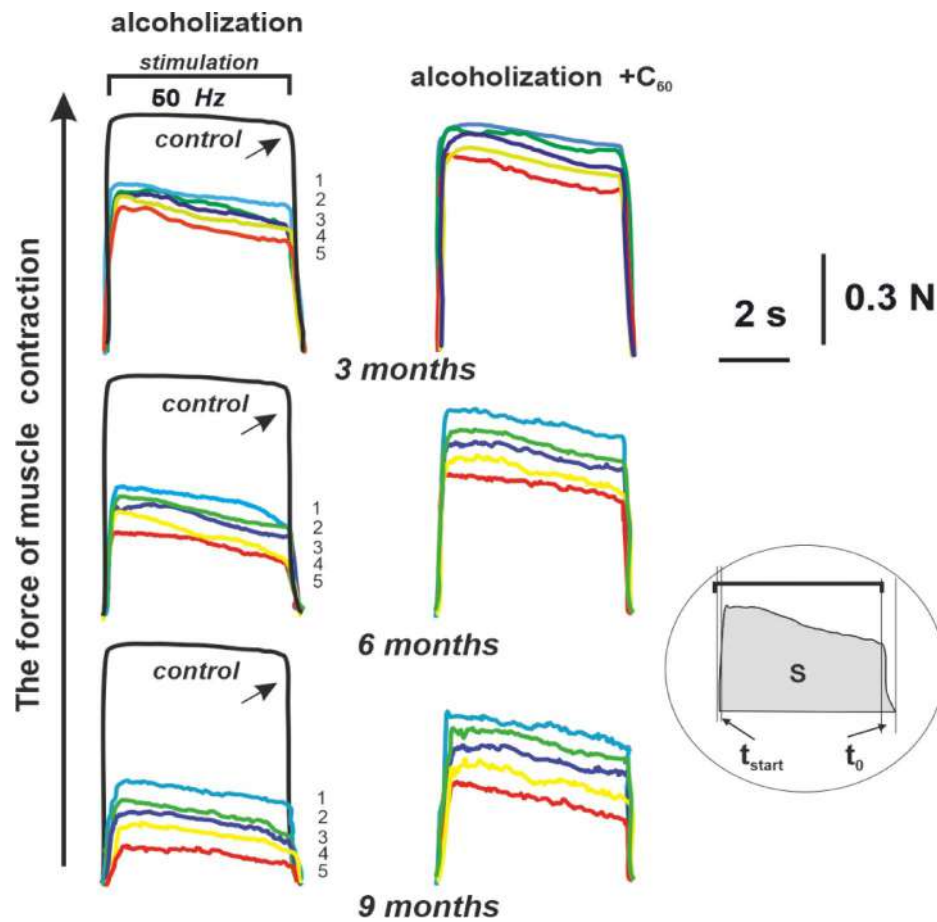


Fig. 1. The strength (N, Newton) of 5 (1, 2, 3, 4, 5) consecutive contractions of the *musculus gastrocnemius* of experimental rats induced by non-relaxation stimulation pools of 50 Hz for 5 s: control – rats of the control group; alcoholization – alcohol-exposed rats; alcoholization + C_{60} – administration of C_{60} fullerene mixed with alcohol; 3, 6, 9 months – duration of the experiment 3, 6 and 9 months, respectively; S – integrated muscle power (an area under the force curve); t_{start} – time of the beginning of the muscle force response; t_0 – time for the muscle force response to reach its initial level.

the use of C_{60} fullerenes, this parameter decreased to 570 ± 9 , 680 ± 11 , and 710 ± 10 ms, respectively, which was $17 \pm 1\%$, $24 \pm 1\%$, and $41 \pm 3\%$ compared to the alcohol-exposed group.

To analyze the time for the muscle contraction force to reach the maximum level, we used a complex modulated stimulation signal with an extended time of the pre-tetanic phase of contraction lasting 3 s (Fig. 5a).

As illustrated in Fig. 5b, in the control group, the time for the muscle contraction force to reach its maximum level (3120 ± 28 ms) was consistent with the duration of pre-tetanic stimulation. After AUD for 3, 6, and 9 months, this parameter was 4530 ± 46 , 5515 ± 52 , and 6150 ± 55 ms, respectively. With the use of C_{60} fullerenes, it decreased to 3300 ± 29 , 3820 ± 32 and 4200 ± 41 ms, respectively, which was $27 \pm 1\%$, $31 \pm 2\%$, and $32 \pm 2\%$ compared to the alcohol-exposed group.

Biochemical analysis of blood in alcohol-exposed rats

To verify the antioxidant effect of C_{60} fullerenes through modification of ROS-dependent mechanisms and their effect on the activity of endogenous antioxidants, we analyzed the values of pro- and antioxidant balance (superoxide dismutase (SOD) and catalase (CAT) activities, reduced glutathione (GSH) concentration) in the blood plasma of alcohol-exposed rats (Fig. 6).

SOD is the most powerful natural antioxidant and the first-line enzyme of the body's antioxidant defense. As can be seen from Fig. 6, the activity of SOD increased to 3.8 ± 0.3 , 4.8 ± 0.4 and 5.9 ± 0.5 Units/ml in the case of 3, 6, and 9 months of AUD, respectively, and in the control it was 1.7 ± 0.1 Units/ml. The use of C_{60} fullerenes reduced these indicators to 2.4 ± 0.2 , 3.0 ± 0.3 and 3.6 ± 0.3 Units/ml, respectively, which amounted to $37 \pm 3\%$, $38 \pm 3\%$, and $39 \pm 3\%$ compared to the alcohol-exposed group.

CAT works in conjunction with SOD by decomposing hydrogen peroxide into water and molecular oxygen, thereby preventing the formation of highly reactive hydroxyl radicals. The CAT activity increased to 2.2 ± 0.1 , 2.5 ± 0.1 and 3.5 ± 0.2 mM/min in the case of 3, 6, and 9 months of AUD, respectively, and in the control group it was 1.3 ± 0.1 mM/min. The use of C_{60} fullerenes reduced these values to 1.7 ± 0.1 , 1.9 ± 0.1 and 2.2 ± 0.1 mM/min, respectively, which amounted to $33 \pm 3\%$, $34 \pm 3\%$, and $37 \pm 3\%$ compared to the alcohol-exposed group (Fig. 6).

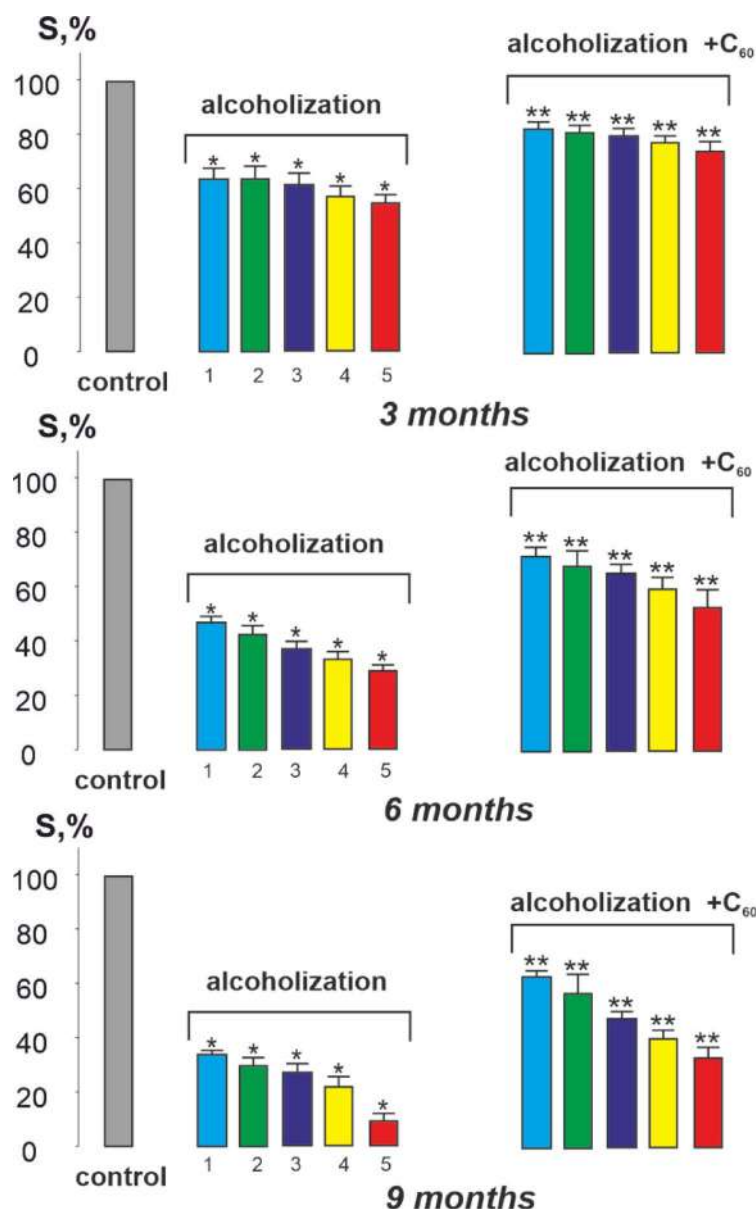


Fig. 2. Integrated power of the *musculus gastrocnemius* (S): control – rats in the control group (S taken as 100%); alcoholization – alcohol-exposed rats; alcoholization + C₆₀ – administration of C₆₀ fullerene mixed with alcohol; 3, 6, 9 months – duration of experiment 3, 6 and 9 months, respectively; 1, 2, 3, 4, 5 – numbering of 5 consecutive contractions of the *muscle gastrocnemius*. * $p < 0.05$ vs. control group ($n = 36$)); ** $p < 0.05$ vs. alcohol-exposed group ($n = 36$; $n = 12$ animals in each subgroup: 3, 6, and 9 months).

The cellular mechanisms of antioxidant defense are linked to the functioning of a powerful GSH link. Figure 6 shows that there is a significant increase in the concentration of GSH in the development of CAM, namely, it was 3.2 ± 0.2 , 3.9 ± 0.2 and 5.5 ± 0.3 μM in the case of 3, 6, and 9 months of AUD, respectively, and in the control – 2.0 ± 0.1 μM . The use of C₆₀ fullerenes reduced these indicators to 2.8 ± 0.1 , 3.0 ± 0.2 and 3.8 ± 0.2 μM , respectively, which amounted to $22 \pm 1\%$, $23 \pm 1\%$, and $31 \pm 3\%$ compared to the alcohol-exposed group.

Analysis of molecular dynamics (MD) simulation

Initially, MD simulations were performed on systems containing 1 to 4 C₆₀ fullerenes in ethanol solutions. In these cases, complexes from 2 to 4 C₆₀ molecules were destroyed with the appearance of single C₆₀ fullerenes during simulation. Single C₆₀ fullerenes don't adsorb molecules of ethanol. The other data can be seen for the cases with 5, 10, 13, and 20 C₆₀ fullerene units. These numbers of C₆₀ fullerene created the stable, predominantly ellipsoidal nanoaggregates that don't break down in MD running in the ethanol solution. Similar results are observed for C₆₀ fullerenes in polar solvents^{30–32}. 5 C₆₀ fullerenes are placed at the tops of a triangular bipyramid, the maximal diagonal of which is 2.2 nm. Nanoaggregates consisting of 10 and 13 C₆₀ fullerenes are almost spherical with diameters of 2.5 and 2.9 nm, respectively. The C₆₀ fullerene cluster, constructed from 20 units,

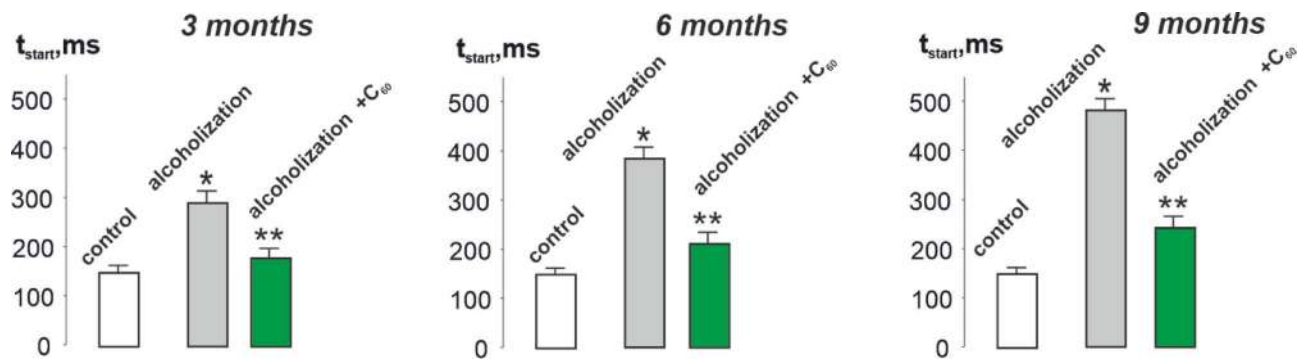


Fig. 3. Time of the beginning of *musculus gastrocnemius* force response (t_{start}): control – rats of the control group; alcoholization – alcohol-exposed rats; alcoholization + C_{60} – administration of C_{60} fullerene mixed with alcohol; 3, 6, 9 months – duration of the experiment 3, 6 and 9 months, respectively. * $p < 0.05$ vs. control group ($n = 36$); ** $p < 0.05$ vs. alcohol-exposed group ($n = 36$; $n = 12$ animals in each subgroup: 3, 6 and 9 months).

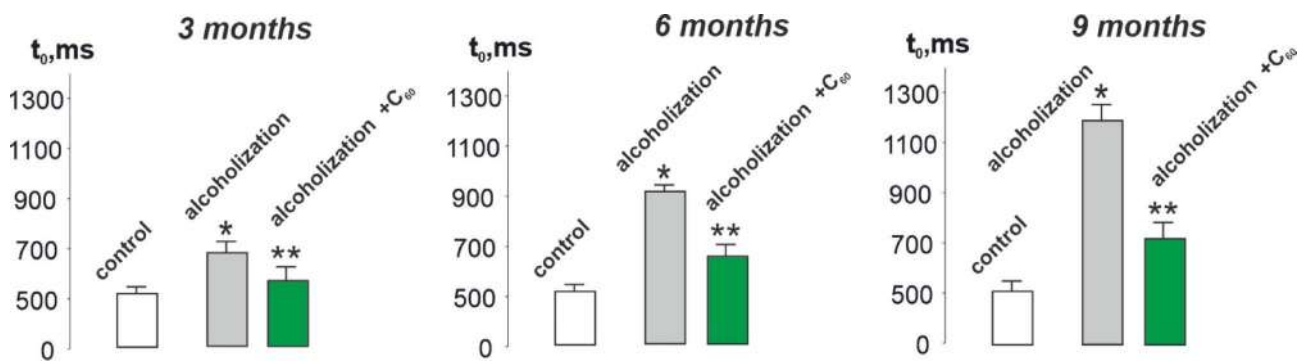


Fig. 4. Time for the *musculus gastrocnemius* force response to reach its initial level (t_0): control – rats of the control group; alcoholization – alcohol-exposed rats; alcoholization + C_{60} – administration of C_{60} fullerene mixed with alcohol; 3, 6, 9 months – duration of the experiment 3, 6 and 9 months, respectively. * $p < 0.05$ vs. control group ($n = 36$); ** $p < 0.05$ vs. alcohol-exposed group ($n = 36$; $n = 12$ animals in each subgroup: 3, 6, and 9 months).

has an ellipsoidal shape with the longest principal axis of 3.4 nm and two others approximately equal to 2.5 nm. Distances represented here were measured between the farthest points of opposite C_{60} fullerene surfaces.

The minimal distance of the neighboring C_{60} fullerenes in clusters, directly interact with each other is 0.27 nm, and the maximum is 0.32 nm. The number of contacts between the C_{60} fullerene cluster and ethanol/water molecules at a distance of 0.32 nm (Fig. 7) was calculated. The averaged values of these contacts are represented in Table 1. On average, the number of contacts between clusters and water is only 23% larger than with ethanol, which means that during simulations, nanoaggregates adsorbed on their surface both ethanol and water molecules. Figure 8a–d shows the first solvation shell of C_{60} fullerene nanoaggregates consisting of 5, 10, 13, and 20 units. The shortest distance between the surface of C_{60} fullerene clusters and ethanol molecules is 0.24 nm, a few water molecules can touch some C_{60} fullerenes at a distance of 0.22 nm. Hence, ethanol molecules occupy clusters inhomogeneously: they tend to be located in the cavities between C_{60} fullerenes, whereas water mostly interacts with the convex surfaces of C_{60} fullerenes. Also, it is determined that some “dry” regions (Fig. 8), which is confirmed by conclusions in the study³³.

Moreover, the majority of ethanol OH-groups orient to water molecules, tending to create hydrogen bonds with them (Fig. 8e). The net of these hydrogen bonds becomes thicker with the increase of the cluster size. In general, the amount of adsorbed ethanol molecules depends on the measure of C_{60} fullerene nanoaggregates (Fig. 7; Table 1).

Discussion

The results obtained from measuring the weight of the experimental animals showed that alcohol consumption reduced their weight gain to $15 \pm 1\%$ compared to the control group, which agrees well with previous data¹⁰. The use of water-soluble C_{60} fullerenes did not affect this process.

The biomechanical studies conducted revealed that the value of integrated muscle power (Fig. 2) decreases, and the values of the time characteristics of *musculus gastrocnemius* contraction (Figs. 3, 4 and 5) increase, in the context of chronic alcohol intoxication in animals. These effects may be caused by impaired interaction of muscle myofilaments, an increase in their stiffness, dysfunction of the calcium pump, and the sarcoplasmic reticulum

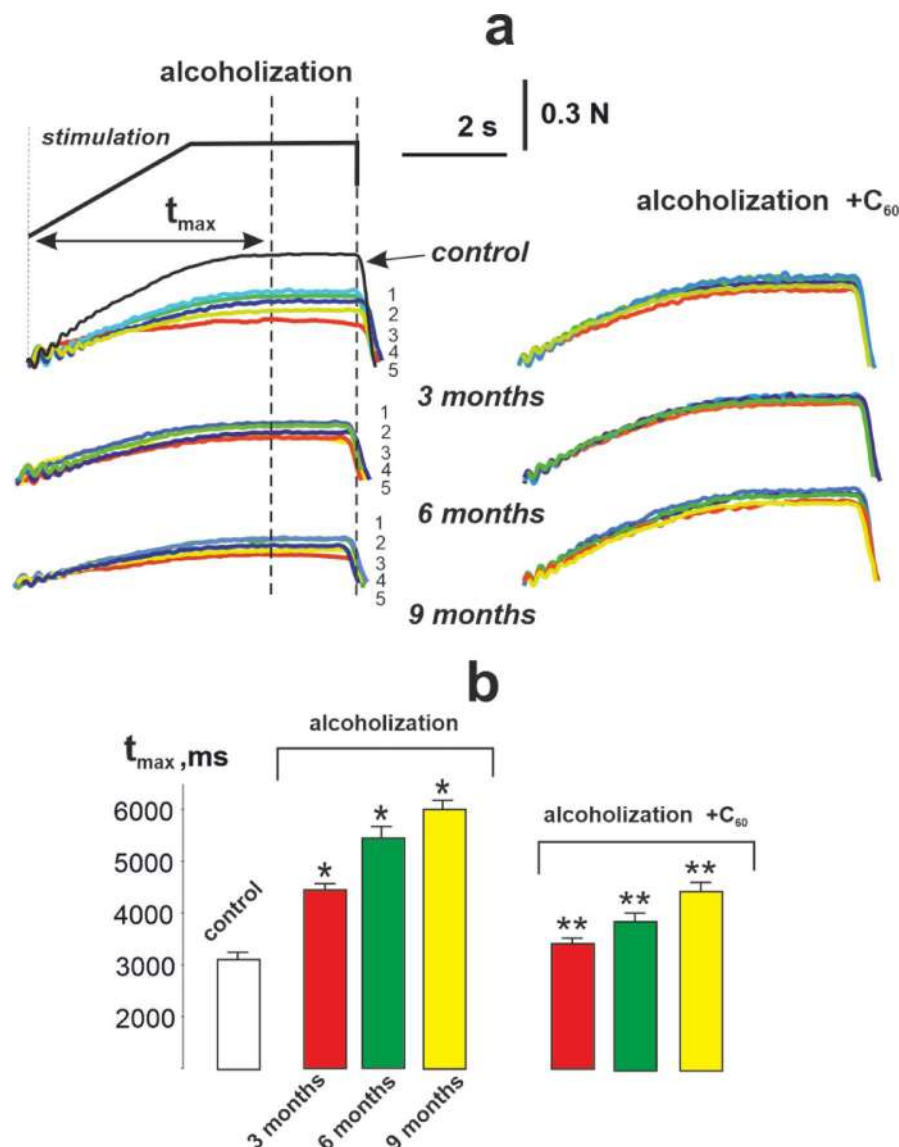


Fig. 5. Mechanograms depicting the force response (N, Newton) of the *musculus gastrocnemius* of experimental rats elicited by 5 s of non-relaxation stimulation pools with an extended pre-tetanic contraction phase (a); $t_{\max}$ – time for the muscle contraction force to reach the maximum level (b): control – rats in the control group; alcoholization – alcohol-exposed rats; alcoholization + C₆₀ – administration of C₆₀ fullerene in conjunction with alcohol; 3, 6, 9 months – duration of experiment 3, 6 and 9 months, respectively; 1, 2, 3, 4, 5 – numbering of 5 consecutive contractions of the *musculus gastrocnemius*. * $p < 0.05$ vs. control group ($n = 36$); ** $p < 0.05$ vs. alcohol-exposed group ($n = 36$; $n = 12$ animals in each subgroup: 3, 6, and 9 months).

system^{5,34,35}. One of the reasons for the above-described mechanokinetic effects may also be a decrease in the synthesis of myotic proteins. It has been shown³⁶ that in the case of CAM, there is a decrease in the synthesis of not only the main contractile protein, myosin, but also cytoskeletal proteins (actin, troponin, titin, nebulin), which provide conditions for the interaction of actin and myosin. This not only changes the stiffness of myocytes but also extends the time for establishing a smooth tetanic muscle contraction. Furthermore, the use of water-soluble C₆₀ fullerenes improved the studied biomechanical parameters by $16\text{--}50 \pm 3\%$ compared to the alcohol-exposed group. Thus, C₆₀ fullerenes can mitigate damage to the main links of the “muscle excitation-response”, inhibiting the development of pathological processes in alcohol-dependent muscle cell damage.

The conducted biochemical studies showed (Fig. 6) that the blood plasma biomarkers associated with oxidative stress in experimental animals exhibited a significant increase against the background of CAM development. This indicates that the muscular system performs work that is too intense for its physiological level. The use of C₆₀ fullerenes, possessing a strong antioxidant capability, improved the biochemical parameters by $22\text{--}39 \pm 2\%$ compared to the alcohol-exposed group. Therefore, the restoration of biochemical indicators achieved through C₆₀ fullerene intervention demonstrates its capacity to disrupt the cycle of oxidative stress and mitochondrial dysfunction.

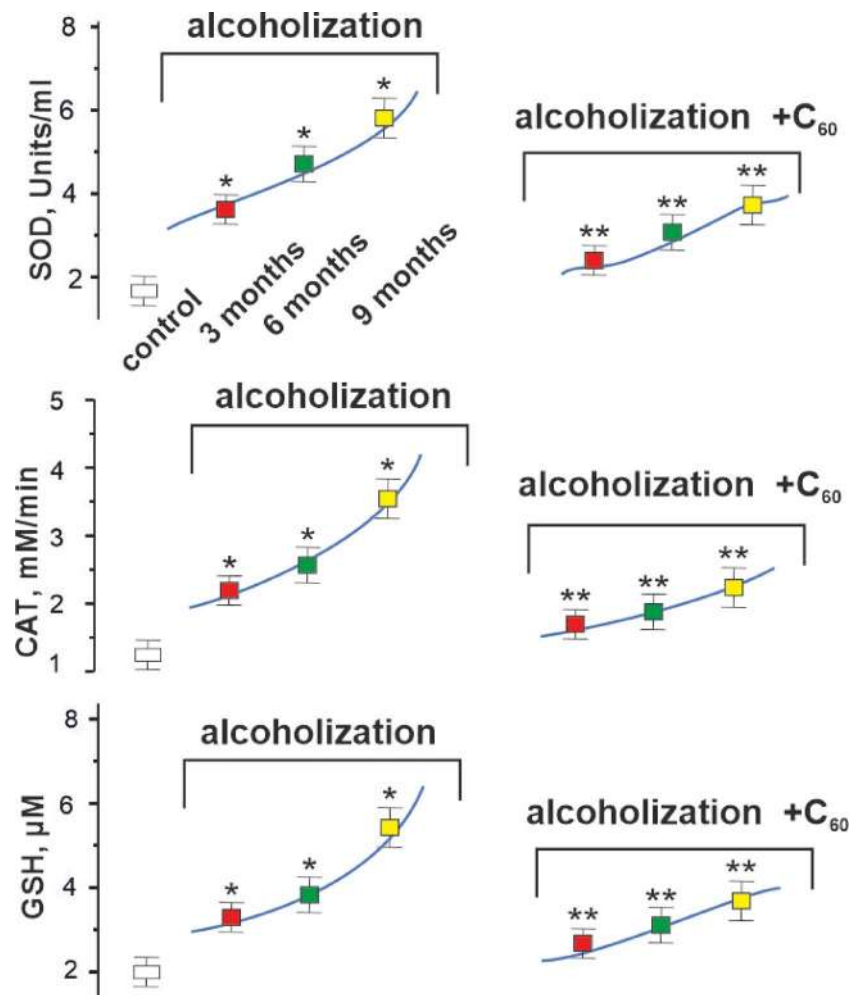


Fig. 6. Parameters of pro- and antioxidant balance (SOD, CAT, and GSH) in the blood plasma of experimental rats: control – rats of the control group; alcoholization – alcohol-exposed rats; alcoholization + C₆₀ – administration of C₆₀ fullerene in a mixture with alcohol; 3, 6, 9 months – duration of the experiment 3, 6 and 9 months, respectively. * $p < 0.05$ vs. control group ($n = 36$); ** $p < 0.05$ vs. alcohol-exposed group ($n = 36$; $n = 12$ animals in each subgroup: 3, 6, and 9 months).

Finally, in addition to the antioxidant effect of C₆₀ fullerenes, one of the explanations for their effective use in combination with alcohol may be the binding of ethanol molecules to C₆₀ fullerene nanoparticles, excluding them from the metabolic cycle and thus reducing the development of CAM and improving the skeletal muscle contraction, which was confirmed by the model calculations (Fig. 8).

The mechanisms underlying the toxic effects of alcohol mediated by oxidative stress are complex and not yet fully elucidated. Ethanol has been shown to induce oxidative damage, including lipid peroxidation (LPO) of cellular membranes and mitochondrial dysfunction³⁷. Ethanol metabolism involves several enzymatic pathways, including alcohol dehydrogenase, the microsomal ethanol oxidizing system (MEOS), and catalase. Each of these pathways generates free radicals that can compromise the body's antioxidant defense system. Additionally, ethanol itself, hyperlactacidemia, and elevated NADH levels enhance xanthine oxidase activity, leading to increased superoxide production. Both LPO and superoxide generation are correlated with cytochrome P450 2E1 expression. MEOS contributes to oxidative stress both directly and indirectly by disrupting endogenous protective mechanisms³⁸. These multifaceted systemic processes hinder the identification of the most influential factors in the development of CAM.

It has been demonstrated that incorporating the antioxidant NAC into the diet of alcohol-exposed rats enhances the activity of glutathione peroxidase and citrate synthase, thereby improving energy metabolism³⁹. The addition of the antioxidant procysteine mitigated alcohol-induced oxidative stress compared to untreated rats⁴⁰. In contrast, the addition of alpha-tocopherol did not prevent the decline in muscle protein synthesis in animals administered alcohol³⁴. The use of well-known antioxidants such as carotenoids, vitamins C and E, selenium, polyphenols, and flavonoids to normalize oxidative stress in skeletal muscles affected by alcohol also yielded conflicting results⁴¹.

In summary, the following potential mechanisms of C₆₀ fullerene action can be considered. Ethanol disperses from the bloodstream to all tissues and body fluids⁴². The ethanol concentration in a tissue is influenced by

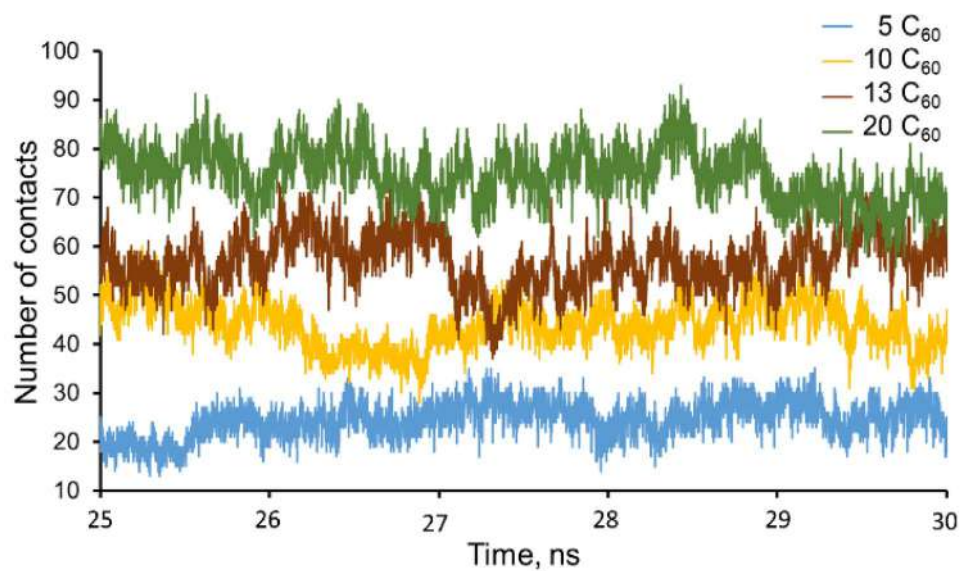


Fig. 7. Contact numbers of the first solvation shell with C₆₀ fullerene nanoaggregates during the last 5 ns of simulation.

Number of C ₆₀ fullerenes in nanoaggregates	Average number of ethanol - C ₆₀ fullerene contacts	Average number of water - C ₆₀ fullerene contacts
5	25 ± 4	29 ± 6
10	44 ± 5	67 ± 9
13	55 ± 6	74 ± 11
20	75 ± 6	93 ± 11

Table 1. Average numbers of ethanol - C₆₀ fullerene and water - C₆₀ fullerene contacts (M ± SE).

its relative water content. Similar to water, ethanol can permeate cell membranes⁴³. Additionally, there are no proteins in blood plasma that bind to ethanol⁴⁴. It is known that more than 90% of consumed alcohol is metabolized by oxidative and non-oxidative pathways, producing such reactive compounds as acetaldehyde, acetate, fatty acid ethyl ester, etc^{18,45}. Within the framework of such processes, ROS is generated, which causes an increase in oxidative stress and LPO, thus disrupting the structural integrity of cells and the functions of tissues and organs. Increased ROS production reduces the level of endogenous antioxidants⁴⁶. Therefore, it is assumed that, on the one hand, C₆₀ fullerenes, as powerful exogenous antioxidants^{19,21} can effectively absorb ROS and remove them from the body⁴⁷ normalizing the functional state of the muscular system against the background of alcoholic myopathy. On the other hand, stable-sized nanoparticles of C₆₀ fullerenes present in ethanol can effectively bind its molecules and, thus, entering the body, reduce the negative effects of alcohol on the functioning of the muscular system.

Materials and methods
Preparation of water-soluble C₆₀ fullerene

A water-soluble C₆₀ fullerene with purity of more than 99.95% has been prepared according to the reproducible method^{48,49}, which is based on transferring C₆₀ molecules from toluene solution into the aqueous phase by ultrasonic treatment. The used concentration of C₆₀ fullerenes in water was 0.15 mg/ml. According to the data of high-resolution microscopy and light scattering spectroscopy⁴⁸ the resulting sample is a typical colloidal nanofluid, which mainly contains both individual C₆₀ fullerenes and their nanoaggregates up to 100 nm. Moreover, it remains highly stable for up to 18 months at a temperature range of +4–25 °C.

In vivo study

The rats come from the university’s breeding program (Institute of Biology and Medicine). All experiments were conducted on male Wistar rats aged 1 month with a body weight of 170 ± 10 g^{26,27,50}. The rats were housed under controlled environmental conditions (21 °C, 12 h light – 12 h dark cycle) with free access to water and a standard diet *ad libitum*. It is important to note that the selection of these animals was based on the following considerations: (1) to eliminate the influence of cyclical physiological processes occurring in female rats, which could potentially affect the results of a long-term study (lasting up to 9 months), we utilized male rats as the experimental subjects; (2) the experimental studies on male Wistar rats commenced immediately after their sexual maturation, which occurs between 4 and 6 weeks of age; (3) previously published experimental data¹³

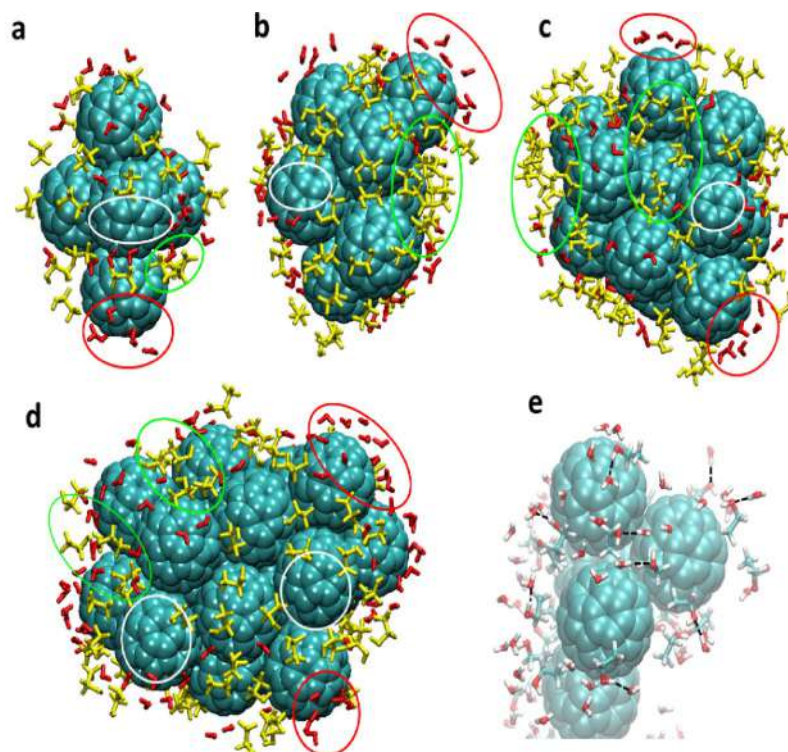


Fig. 8. The first solvation shell of the 5, 10, 13, and 20 C_{60} fullerene nanoaggregates (a–d). Water molecules are red, ethanol is yellow. Circles show: white – “dry” regions, green – cavities between C_{60} fullerenes, and red – convex surfaces. Fragment of 5 C_{60} fullerene complex with first ethanol solvation shell, where the molecules are occupied by water within 0.24 nm (e). The black dotted lines demonstrate hydrogen bonds between ethanol OH-groups and water. Oxygen atoms are red, hydrogens – white, and carbons – cyan.

indicate that alcohol consumption in animals younger than 1 month does not lead to a statistically significant development of CAM. Each animal was housed individually and provided with a standard diet *ad libitum* in both quantity and composition. Body weight was measured daily for all animals across experimental groups throughout the study using Lab scales (“Dneproves FEH”, Ukraine). All procedures with laboratory animals complied with the ARRIVE guidelines. The use of animals was approved by the Biomedical Ethics Committee of the ESC “Institute of Biology and Medicine” of Taras Shevchenko National University of Kyiv (protocol no. 1 dated January 8, 2024) and performed following Article 26 of the Law of Ukraine “On the Protection of Animals from Cruelty” (no. 3447-IV, 21.02.2006), as well as European Union Directive of 22 September 2010 (2010/63/EU) for the protection of animals used for scientific purposes.

All tested rats were randomly divided into the following experimental groups:

- control ($n = 36$; 12 animals in each subgroup); animals received 100% drinking water;
- alcoholization ($n = 36$). Each rat was placed in a separate cage to receive 40% ethanol in drinking water, which meant that the animals did not have access to 100% water until they had consumed the dosed portion of ethanol⁵¹. The amount of ethanol consumed was calculated for 0.5% of the animal’s body weight. Recalculation of the ethanol dose was performed every 24 h throughout the experiment. The duration of AUD was 3 ($n = 12$), 6 ($n = 12$), and 9 ($n = 12$) months;
- alcoholization + C_{60} ($n = 36$; 12 animals in each subgroup). All tested rats, together with ethanol, were orally administered C_{60} fullerene at daily doses of 1 mg/kg. Recalculation of the C_{60} fullerene dose was performed every 24 h throughout the experiment. Control of the amount of C_{60} fullerene ingested was performed by denying the rats access to drinking 100% water until they fully consumed the applied drug^{26,27,50}.

The dose of C_{60} fullerene was chosen as the most effective based on previous results^{26,27,50}. Moreover, the total dose of 270 mg/kg in the experiment lasting 9 months is lower than the LD_{50} value, which was 600 mg/kg of body weight when administered orally to rats⁵² and 721 mg/kg when administered intraperitoneally to mice⁵³. Also, it is important to note that after intravenous administration to mice, the radiolabeled C_{60} fullerenes predominantly accumulate in the blood, spleen, stomach, and liver and are excreted within 72 h, mainly with urine⁴⁷. Finally, the histopathological and biochemical data show that C_{60} fullerene (at a daily dose of 1 mg/kg for 3, 6, and 9 months) reduces the level of liver damage in chronic alcohol intoxication of rats⁵⁰. Therefore, a daily dose of 1 mg/kg is considered safe for biotesting.

Anesthesia of experimental rats was carried out by intraperitoneal injection of Zoletil (25 mg/kg, Virbac, France).

Experiments were conducted during the light phase, 4 to 5 h after alcohol administration. The interval between the induction of anesthesia and the start of the experiment did not exceed 15 min, and the total duration of each experiment was limited to 1 h.

Euthanasia of animals is conducted by administering an overdose of the anesthetic agent.

Biomechanical and biochemical analyses

The contraction force of the *musculus gastrocnemius* was recorded with the Aurora Scientific (ASI) 305 C-LR Dual-Mode Lever System (USA) tensometric equipment.

In the distal part of the experimental *musculus gastrocnemius*, the tendon portion was cut and attached to the fixation sensors. In the L4-L5 segments, the ventral roots of the efferents were cut directly at their exit points from the spinal cord. The filaments of the severed ventral roots were fixed onto stimulating electrodes, and cyclic distribution of non-relaxation pulses was performed at a frequency of 50 Hz for 5 s. Control of the external load on the muscle was carried out using a system of mechanostimulators^{26,27}.

In the framework of studying the biomechanics of muscle contraction, we analyzed the following indicators as markers of muscle dysfunction²⁵:

1. integrated muscle power (S) is an area under the force curve, which is an indicator of the overall performance of the muscle under the applied stimulation pools. This value was calculated numerically using Origin 9.4 software and taken as 100% for the control group;
2. time of the beginning of the muscle force response (latent period; t_{start}). This parameter indicates the level of pathological changes at the stages of the interaction of muscle myofilaments, dysfunction of the calcium pump, and the sarcoplasmic reticulum system, which are the result of myocyte membrane disruption;
3. time for the muscle force response to reach its initial level, taken as zero (t_0). The increase in intramuscular collagen structures, the presence of nonfunctional muscle fibers, and the development of inflammation reduce the level of dynamic parameters of muscle contraction. Therefore, this parameter indicates a change in muscle stiffness associated with both an increase in connective tissue components (long-term pathology) and changes in intramuscular pressure (an inflammatory process);
4. time for the muscle contraction force to reach the maximum level (t_{max}). Increasing this parameter triggers the CNS to recruit additional muscle fibers to ensure adequate muscle function. However, this can adversely impact the precise positioning of the joints⁵⁴.

The concentration of GSH, CAT and SOD activities in the blood plasma of experimental rats, as the important markers of oxidative stress in the development of CAM^{55,56} were determined using biochemical analyzers RNL-200 and JN-1101-TR2 (Netherlands).

Blood was collected from the experimental animals via femoral arterial catheterization⁵⁷ and after that analyzed immediately using biochemical analyzers.

Our previous studies⁵⁰ have shown significant pathological changes in the functioning of the organs of alcohol-exposed rats. Therefore, in the case of long-term CAM, the analysis of pro-antioxidant balance in muscle tissue may not be adequate, since during such a long period (up to 9 months), significant multi-organ disorders occur. Thus, we measured the values of biochemical indicators in the blood plasma of experimental rats.

MD simulation

We simulated 1–5, 10, 13, and 20 C_{60} fullerene clusters in ethanol solution. The initial structure of a single C_{60} fullerene was built by Avogadro software⁵⁸. Nanoaggregates with 2–5, 10, 13, and 20 C_{60} fullerene units were constructed using package PackMol⁵⁹. The same recourse was applied to put these clusters into the box with the solution of 1000 ethanol and 23,832 water molecules which corresponds to 11% of alcohol (a calculation model approximation). MD simulations are implemented in NAMD⁶⁰ using force field CHARMM 36 with the type TYP3P of water atoms and Girifalco VDW parameters for C_{60} molecules⁶¹. Periodic boundary conditions are set in all directions. Firstly, C_{60} fullerene – ethanol/water systems were minimized during up to 10 ns in the NVT ensemble. Further systems were run for approximately 30 ns in the NPT ensemble. The size of the simulated box varied depending on the atom number in the systems to control the density of the ethanol solution, which was achieved at 0.98 g/cm³ corresponding to the reference value of 11% alcohol. Analysis of trajectories represented here is for the last 5 ns of running by VMD software (<http://www.ks.uiuc.edu/Research/vmd/>)⁶².

Statistical analysis

The statistical evaluation of the experimental results was performed by the software package Statistica 8.0 (Dell, USA) using the procedure of analysis of variances (ANOVA) with mixed design⁶³. Two between-group factors were supposed: (1) alcoholization (three levels – AUD lasting 3, 6, and 9 months); (2) C_{60} fullerene treatment (two levels – no and use of C_{60} fullerene). The Shapiro-Wilk W-test was used to test for normality. Levene's test was used to assess the equality of variances across groups. Multiple pairwise comparisons between different groups and conditions were performed by Bonferroni *post-hoc* test. The differences between the groups were considered significant at $p < 0.05$. Each of the experimental force curves is the result of averaging 10 similar tests for each animal in the group. Each biochemical measurement was assayed in triplicate.

Conclusion

The obtained results demonstrate a pronounced therapeutic effect of C_{60} fullerenes at the level of 16–50 ± 3% and 22–39 ± 2% for the studied biomechanical parameters of *musculus gastrocnemius* contraction and biochemical indicators of pro- and antioxidant balance in the blood of experimental rats, respectively, compared to the alcohol-exposed group against the background of CAM development. This indicates that the nanoantioxidants

C₆₀ fullerenes, effectively absorbing ROS, can improve the functional state of the muscular system during chronic alcohol intoxication. Finally, model calculations have shown that C₆₀ fullerene nanoparticles present in ethanol, effectively binding its molecules, are also able to reduce the negative effect of alcohol on the functioning of the rat *musculus gastrocnemius*. Thus, C₆₀ fullerenes can be considered promising drugs to reduce the extent of CAM symptoms developed, which requires further clinical trials.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Received: 24 February 2025; Accepted: 19 August 2025

Published online: 23 September 2025

References

1. Volkow, N. D., Gordon, J. A. & Koob, G. F. Choosing appropriate language to reduce the stigma around mental illness and substance use disorders. *Neuropsychopharmacol* **46**, 2230–2232 (2021).
2. Rehm, J. et al. Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. *Lancet* **373**, 2223–2233 (2009).
3. Global status report on alcohol and health. (World Health Organization's, 2024).
4. Anderson, P. & Baumberg, B. *Alcohol in Europe: A Public Health Perspective. A Report for the European Commission*. 446. (Institute of Alcohol Studies, 2006).
5. Preedy, V. R. et al. Alcoholic skeletal muscle myopathy: definitions, features, contribution of neuropathy, impact and diagnosis. *Eur. J. Neurol.* **8**, 677–687 (2001).
6. Fernández-Solà, J., Junyent, J. M. & Urbano-Márquez, A. Alcoholic myopathies. *Curr. Opin. Neurol.* **9**, 400–405 (1996).
7. Vary, T. C., Frost, R. A. & Lang, C. H. Acute alcohol intoxication increases atrogen-1 and MuRF1 mRNA without increasing proteolysis in skeletal muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R1777–1789 (2008).
8. Bourgeois, B. L. et al. Chronic alcohol and skeletal muscle. In *Handbook of Substance Misuse and Addictions: from Biology To Public Health* 943–967 (Springer, 2022).
9. Adler, K., Molina, P. E. & Simon, L. Epigenomic mechanisms of alcohol-induced impaired differentiation of skeletal muscle stem cells; role of class IIA histone deacetylases. *Physiol. Genomics* **51**, 471–479 (2019).
10. Simon, L., Bourgeois, B. L. & Molina, P. E. Alcohol and skeletal muscle in health and disease. *Alcohol Res.* **43**, 04 (2023).
11. Singh, S. et al. Acute alcoholic myopathy, rhabdomyolysis and acute renal failure. *Neurol. India* **48**, 84–85 (2000).
12. Sacanella, E. et al. Chronic alcoholic myopathy: diagnostic clues and relationship with other ethanol-related diseases. *QJM* **88**, 811–817 (1995).
13. Koll, M. et al. Effect of acute and chronic alcohol treatment and their superimposition on lysosomal, cytoplasmic, and proteosomal protease activities in rat skeletal muscle in vivo. *Metabolism* **51**, 97–104 (2002).
14. Moser, S. E. et al. Neuromuscular mechanisms of weakness in a mouse model of chronic alcoholic myopathy. *Alcohol Clin. Exp. Res.* **46**, 1636–1647 (2022).
15. Preedy, V. R. et al. The importance of alcohol-induced muscle disease. *J. Muscle Res. Cell. Motil.* **24**, 55–63 (2003).
16. Otis, J. S. & Guidot, D. M. Procysteine stimulates expression of key anabolic factors and reduces plantaris atrophy in alcohol-fed rats. *Alcohol Clin. Exp. Res.* **33**, 1450–1459 (2009).
17. Kumar, A. et al. Oxidative stress mediates ethanol-induced skeletal muscle mitochondrial dysfunction and dysregulated protein synthesis and autophagy. *Free Radic Biol. Med.* **145**, 284–299 (2019).
18. Wu, D. & Cederbaum, A. I. Alcohol, oxidative stress, and free radical damage. *Alcohol Res. Health* **27**, 277–284 (2003).
19. Goodarzi, S. et al. Fullerene: biomedical engineers get to revisit an old friend. *Mater. Today* **20**, 460–480 (2017).
20. Gaur, M. et al. Biomedical applications of carbon nanomaterials: fullerenes, quantum dots, nanotubes, nanofibers, and graphene. *Materials* **14**, 5978 (2021).
21. Eswaran, S. V. Water soluble nanocarbon materials: a panacea for all? *Curr. Sci.* **114**, 1846–1850 (2018).
22. Vereshchaka, I. V. et al. C₆₀ fullerenes diminish the muscle fatigue in rats comparable to N-acetylcysteine or β-alanine. *Front. Physiol.* **9**, 517 (2018).
23. Foley, S. et al. Cellular localisation of a water-soluble fullerene derivative. *Biochem. Biophys. Res. Commun.* **294**, 116–119 (2002).
24. Nozdrenko, D. M. et al. Protective effect of water-soluble C₆₀ fullerene nanoparticles on the ischemia-reperfusion injury in the muscle soleus of rat. *Int J. Mol. Sci.* **22**, 6812 (2021).
25. Nozdrenko, D. et al. C₆₀ fullerene protective effect against the rat muscle soleus trauma. *Heliyon* **10**, e32677 (2024).
26. Motuziuk, O. et al. C₆₀ fullerene reduces the level of fluctuations in the force response of muscle gastrocnemius in chronically alcoholized rats. *Appl. Nanosci.* **13**, 7057–7067 (2023).
27. Motuziuk, O. et al. The effect of C₆₀ fullerene on the mechanokinetics of muscle gastrocnemius contraction in chronically alcoholized rats. *Heliyon* **9**, e18745 (2023).
28. Crowell, K. T., Laufenberg, L. J. & Lang, C. H. Chronic alcohol consumption, but not acute intoxication, decreases in vitro skeletal muscle contractile function. *Alcohol Clin. Exp. Res.* **43**, 2090–2099 (2019).
29. Nozdrenko, D. M. et al. C₆₀ fullerene as promising therapeutic agent for the prevention and correction of functioning skeletal muscle at ischemic injury. *Nanoscale Res. Lett.* **12**, 115 (2017).
30. Fortner, J. D. et al. C₆₀ in water: nanocrystal formation and microbial response. *Environ. Sci. Technol.* **39**, 4307–4316 (2005).
31. McHedlov-Petrosyan, N. O. Fullerenes in liquid media: an unsettling intrusion into the solution chemistry. *Chem. Rev.* **113**, 5149–5193 (2013).
32. Guskova, O. A., Varanasi, S. R. & Sommer, J. U. C₆₀-dyad aggregates: Self-organized structures in aqueous solutions. *J. Chem. Phys.* **141**, 144303 (2014).
33. Varanasi, S. R., Guskova, O. A., John, A. & Sommer, J. U. Water around fullerene shape amphiphiles: A molecular dynamics simulation study of hydrophobic hydration. *J. Chem. Phys.* **142**, 224308 (2015).
34. Preedy, V. R. et al. Free radicals in alcoholic myopathy: indices of damage and preventive studies. *Free Radic Biol. Med.* **32**, 683–687 (2002).
35. Souza-Smith, F. M. et al. Physiological processes underlying organ injury in alcohol abuse. *Am. J. Physiol. Endocrinol. Metab.* **311**, E605–619 (2016).
36. Hunter, R. J. et al. Alcohol affects the skeletal muscle proteins, Titin and Nebulin in male and female rats. *J. Nutr.* **133**, 1154–1157 (2003).
37. Oliveira de Araújo Melo, C. et al. Evaluation of polymorphisms in repair and detoxification genes in alcohol drinkers and non-drinkers using capillary electrophoresis. *Electrophoresis* **41**, 254–258 (2020).
38. Zima, T. et al. Oxidative stress, metabolism of ethanol and alcohol-related diseases. *J. Biomed. Sci.* **8**, 59–70 (2001).

39. Seiva, F. R. et al. Alcoholism and alcohol abstinence: N-acetylcysteine to improve energy expenditure, myocardial oxidative stress, and energy metabolism in alcoholic heart disease. *Alcohol* **43**, 649–656 (2009).
40. Otis, J. S. & Guidot, D. M. Procyteine increases alcohol-depleted glutathione stores in rat plantaris following a period of abstinence. *Alcohol Alcohol* **45**, 495–500 (2010).
41. Silva, C. S., Portari, G. V. & Vannucchi, H. Antioxidant treatment and alcoholism. In *Molecular Aspects of Alcohol and Nutrition* 119–131 (Elsevier, 2016).
42. Mukherjee, S., Das, S. K. & Vasudevan, D. M. Effects of ethanol consumption on different organs – A brief overview. *Asian J. Biochem.* **2**, 386–394 (2007).
43. Murray, R. K. & Kennelly, P. J. Ethanol intoxication. In *Harper's Illustrated Biochemistry* 629–647 (McGraw-Hill, (2009).
44. Masters, S. B. The alcohol. In *Basic and Clinical Pharmacology* 387–398 (McGraw-Hill, (2010).
45. Schukit, M. A. Ethanol and methanol. Chapter 23. In *Goodman and Gillman's Manual Pharmacology and Therapeutics* 629–647 (McGraw-Hill, (2014).
46. Ha, H. L., Shin, H. J., Feitelson, M. A. & Yu, D. Y. Oxidative stress and antioxidants in pathogenesis. *World J. Gastroenterol.* **16**, 6035–6043 (2010).
47. Nikolic, N. et al. Preparation and biodistribution of radiolabeled fullerene C₆₀ nanocrystals. *Nanotechnol* **20**, 385102 (2009).
48. Burlaka, A. P. et al. Catalytic system of the reactive oxygen species on the C₆₀ fullerene basis. *Exp. Oncol.* **26**, 326–327 (2004).
49. Ritter, U. et al. Structural features of highly stable reproducible C₆₀ fullerene aqueous colloid solution probed by various techniques. *Fullerenes Nanotubes Carbon Nanostruct.* **23**, 530–534 (2015).
50. Motuziuk, O. et al. C₆₀ fullerene reduces the level of liver damage in chronic alcohol intoxication of rats. *Molecules* **29**, 2951 (2024).
51. Song, K. et al. Chronic ethanol consumption by mice results in activated splenic T cells. *J. Leukoc. Biol.* **72**, 1109–1116 (2002).
52. Gharbi, N. et al. [60]fullerene is a powerful antioxidant in vivo with no acute or subacute toxicity. *Nano Lett.* **5**, 2578–2585 (2005).
53. Prylutska, S. V. et al. In vitro and in vivo toxicity of pristine C₆₀ fullerene aqueous colloid solution. *Fullerenes Nanotubes Carbon Nanostruct.* **27**, 715–728 (2019).
54. Kostyukov, A. I. et al. Effects in feline gastrocnemius-soleus motoneurons induced by muscle fatigue. *Exp. Brain Res.* **163**, 284–294 (2005).
55. Brancaccio, P., Lippi, G. & Maffulli, N. Biochemical markers of muscular damage. *Clin. Chem. Lab. Med.* **48**, 757–767 (2010).
56. Tsermpini, E. E., Ilješ, A. P. & Dolžan, V. Alcohol-induced oxidative stress and the role of antioxidants in alcohol use disorder: A systematic review. *Antioxidants* **11**, 1374 (2022).
57. Jespersen, B., Knupp, L. & Northcott, C. A. Femoral arterial and venous catheterization for blood sampling, drug administration and conscious blood pressure and heart rate measurements. *J. Vis. Exp.* **59**, 3496 (2012).
58. Hanwell, M. D. et al. Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. *J. Cheminform.* **4**, 17 (2012).
59. Martínez, J. M. & Martínez, L. Packing optimization for automated generation of complex system's initial configurations for molecular dynamics and Docking. *J. Comp. Chem.* **24**, 819–825 (2003).
60. Phillips, J. C. et al. Scalable molecular dynamics on CPU and GPU architectures with NAMD. *J. Chem. Phys.* **153**, 044130 (2020).
61. Girifalco, L. A. Interaction potential for carbon (C₆₀) molecules. *J. Phys. Chem.* **95**, 5370–5371 (1991).
62. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. *J. Mol. Graph.* **14**, 33–38 (1996).
63. Prylutsky, Y. et al. C₆₀ fullerene improves the contractile activity of the injured rat muscle gastrocnemius. *Nanotechnol* **36**, 125101 (2025).

Acknowledgements

We acknowledge support for the publication costs by the Open Access Publication Fund of the Technical University of Ilmenau, Germany. The authors are sincerely grateful to Dr. Olga Guskova for the tentative discussion as well as to TU Dresden for the computing resources.

Author contributions

Conceptualization, O.M., D.N. and Y.P.; Methodology, O.M., D.N., S.P., N.T. and S.V.; Formal analysis, S.V. and P.S.; Investigation, O.M., D.N., S.P. and N.T.; Resources, N.T. and P.S.; Data curation, N.T. and S.P.; Writing – original draft, O.M., D.N., M.P. and Y.P.; Writing – review & editing, M.P., Y.P. and U.R.; Project administration, P.S. and U.R.; Supervision, Y.P.; All authors reviewed and approved the manuscript.

Funding

Open Access funding enabled and organized by Projekt DEAL.

Declarations

Ethics declarations

All procedures with laboratory animals complied with the ARRIVE guidelines. The study protocol was approved by the Biomedical Ethics Committee of the ESC “Institute of Biology and Medicine” of Taras Shevchenko National University of Kyiv (protocol no. 1 dated January 8, 2024) and performed following Article 26 of the Law of Ukraine “On the Protection of Animals from Cruelty” (no. 3447-IV, 21.02.2006), as well as European Union Directive of 22 September 2010 (2010/63/EU) for the protection of animals used for scientific purposes.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to U.R.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2025