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НОЗДРЕНКО ДМИТРО МИКОЛАЙОВИЧ

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ДИСЕРТАЦІЯ

Механокінетика скорочення скелетних м’язів за експериментальних патологій та дії вуглецевих наночастинок

03.00.02 - біофізика

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

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АНОТАЦІЯ

Ноздренко Д.М. Механокінетика скорочення скелетних м’язів за експериментальних патологій та дії вуглецевих наночастинок – дисертація на здобуття наукового ступеня доктора біологічних наук за спеціальністю 03.00.02 - біофізика – Київський національний університет імені Тараса Шевченка, Київ, 2023.

Представлена робота присвячена комплексному дослідженню проблеми визначення рівня міопатичних пошкоджень в рамках аналізу механокінетичних параметрів скорочення скелетного м’яза (зокрема, значення мінімальної (F_{min}) і максимальної (F_{max}) сили скорочення м’яза та імпульсу м’язової сили (S), час досягнення максимальної силової відповіді (t_{max}), зменшення сили скорочення м'яза на 50% від початкового рівня (t_{50}), виходу силових параметрів на початковий рівень (t_0) і початку силової відповіді м'яза (t_{start})). За використання сучасних методів тензометрії, біохімічного та гістологічного аналізів детально досліджено зміни біомеханічних маркерів скорочення пошкоджених м’язів щурів, біохімічних показників їх крові та морфології тканин скелетного м'яза за різних експериментально-індукованих м’язових патологій (зокрема, ішемія, механічна травма, втома і атрофія) різного ступеня тяжкості та дії водорозчинних вуглецевих наночастинок - C_{60} фулеренів у різних часових (1-45 діб) і дозових (0,5, 1 і 2 мг/кг) діапазонах залежно від способу (внутрішньом'язове, пероральне) та схеми їх застосування (до і після ініціація м’язової патології).

Розроблено оригінальний алгоритм використання універсальних біомеханічних маркерів амплітудно-швидкісних змін силової відповіді м’яза для аналізу перебігу патологічних процесів у ньому. Доведено, що зменшення ступеня тяжкості патологічного стану м’яза за дії водорозчинних C_{60} фулеренів відображає послідовність «спрацьовування» запропонованих біомеханічних маркерів у такому хронологічному порядку: F_{min} - t_{max} - F_{max} - S - t_{50} - t_{start} - t_0. Це відкриває можливість використання цього алгоритму для визначення рівня м’язового пошкодження при

контролі ефективності терапевтичних і реабілітаційних процедур. Продемонстровані на експериментальних тваринах ефекти дії водорозчинних C_{60} фулеренів сприятимуть створенню ефективних лікарських засобів на їх основі, здатних зменшувати ступінь тяжкості патологій м'язової системи як в стані їх гострого перебігу, так і хронічних наслідків.

Ключові слова: скелетний м’яз, патології м’язової системи, C_{60} фулерен, механокінетичні параметри скорочення м’яза, біохімічний та гістологічний аналізи.

SUMMARY

Nozdrenko D.M. Mechanokinetics of skeletal muscle contraction under experimental pathologies and the action of carbon nanoparticles – dissertation for the scientific degree of Doctor of Biological Sciences in speciality 03.00.02 - biophysics – Taras Shevchenko National University of Kyiv, Kyiv, 2023.

The present work is devoted to a comprehensive study of the problem of determining the level of myopathic damage in the framework of the analysis of the mechanokinetic parameters of skeletal muscle contraction (in particular, the values of the minimum (F_{min}) and maximum (F_{max}) muscle contraction force and muscle force impulse (S), time to reach the maximum force response (t_{max}), decrease in muscle contraction force by 50% of the initial level (t_{50}), return of force parameters to the initial level (t_0) and the beginning of the muscle force response (t_{start})). Using modern methods of strain gauging, biochemical and histological analyses, we studied in detail the changes in biomechanical markers of contraction of damaged rat muscles, biochemical parameters of their blood and skeletal muscle tissue morphology under various experimentally induced muscle pathologies (in particular, ischemia, mechanical trauma, fatigue and atrophy) of varying severity and the effect of water-soluble carbon nanoparticles - C_{60} fullerenes in different time (1-45 days) and dose (0.5, 1 and 2 mg/kg) ranges depending on the method (intramuscular, oral) and scheme of their application (before and after initiation of muscle pathology).

An original algorithm for the use of universal biomechanical markers of amplitude-velocity changes in the force response of the muscle to analyze the course of pathological processes in it has been developed. It is proved that the decrease in the severity of the pathological condition of the muscle under the influence of water-soluble C_{60} fullerenes reflects the sequence of "triggering" of the proposed biomechanical markers in the following chronological order: F_{min} - t_{max} - F_{max} - S - t_{50} - t_{start} - t_0. This opens up the possibility of using this algorithm to determine the level of muscle damage when monitoring the effectiveness of therapeutic and rehabilitation procedures. The effects of water-soluble C_{60} fullerenes demonstrated in experimental animals will contribute to the creation of effective drugs based on them, capable of reducing the severity of pathologies of the muscular system both in the state of their acute course and chronic consequences.

Keywords: skeletal muscle, pathologies of the muscular system, C_{60} fullerene, mechanokinetic parameters of muscle contraction, biochemical and histological analyses.

ЗМІСТ

ПЕРЕЛІК ОСНОВНИХ УМОВНИХ СКОРОЧЕНЬ

АОЗ	антиоксидантна система захисту
АДФ	аденозиндифосфат
АСМ	атомно-силова мікроскопія
АФК	активні форми кисню
АЦП	аналого-цифровий перетворювач
ДРС	динамічне розсіювання світла
КФК	креатинфосфокіназа
ЛДГ	лактатдегідрогеназа
ПОЛ	перекисне окиснення ліпідів
C_{60}FAS	водний розчин C_{60} фулерену
ТБК	тіобарбітурова кислота
ЦАП	цифро-аналоговий перетворювач

ВСТУП

Актуальність дослідження. М'язові патології є одними з найпоширеніших патологій людського організму [Bruce Hamilton, et al., 2017]. На сьогодні відсутня єдина думка щодо способів класифікації та опису їх тяжкості. Проблема визначення рівня міопатичних пошкоджень викликає серйозне занепокоєння через складнощі з прийняттям рішень щодо застосованої терапії, а також високої частоти рецидивів [Hans-Wilhelm, et al., 2013]. Ефективність того чи іншого лікування потребує розуміння молекулярних механізмів, властивих конкретному типу пошкодження скелетних м'язів, та біохімічних процесів, що беруть участь у загоєнні м'язів після травм [Smith, et al., 2019]. Визначення ступеня тяжкості патології біохімічними і гістологічними методами, крім тривалого часу, майже завжди нехтує розвинені побічні ускладнення, що веде до неякісного алгоритму терапевтичних процедур та реабілітаційних заходів [Hurme, et al., 2021]. Таким чином, критично важливою є адекватна ідентифікація м'язових патологій, необхідна для повного розуміння рівня пошкодження та оптимізації процесу його лікування.

Механокінетику цілого м’яза не можна розглядати лише як суму параметрів складових волокон, оскільки його скоротливі властивості залежать значною мірою від розміщення волокон, їх типу, неоднорідності за розмірами та властивостями тощо. У формуванні макропоказників нервово-м'язової активності бере участь велика кількість надзвичайно складних, нелінійних і часто нестаціонарних процесів. Вплив на ці процеси патологічних факторів веде або до повної дисфункції цих параметрів, або їх розсинхронізації [Proske, et al., 2018]. Внаслідок цього цілий м'яз, як динамічна система, не в змозі адекватно реалізовувати пули нейронної активності, що надходять із ЦНС. Характер і рівень цих дисфункцій напряму пов'язані з рівнем розвитку патологічних процесів в організмі, аналіз багатьох з яких можна провести виключно на феноменологічному рівні. Незважаючи на появу нових експериментальних підходів до аналізу процесів нервово-м'язової регуляції на мікрорівні, традиційні електрофізіологічні моделі з використанням нервово-м'язового препарату *in vivo* мають першочергове значення [Nijhof, et al., 2013]. Тому для аналізу змін при

нервово-м'язових патологіях важливий комплексний експериментальний підхід з можливістю одночасного контролю різних біомеханічних параметрів. Лише у цьому випадку з'являється можливість простежити зміни у реакції нервово-м'язових препаратів на стимуляції, що відповідають за розвиток точних позиційних рухів, аналіз яких є важливим при висновку про ефективність застосованого терапевтичного препарату.

Експериментальне вивчення м'язової динаміки традиційно зводиться до визначення трьох взаємозалежних змінних величин:

1) частоти еферентної імпульсації, яка надходить до м'яза;

2) сили, яка розвивається м'язом, рівної за величиною, але протилежної за напрямком до сили зовнішнього впливу на м'яз;

3) довжини м'яза.

Кожна з цих змінних є функцією двох інших і такі функціональні залежності несуть певний фізіологічний зміст. Аналізуючи зміни вхідних параметрів (довжина, навантаження і т.п.), можна визначити лінійною чи нелінійною є досліджувана система, а також, що найбільш важливо, кількісно проаналізувати її властивості (Jacques, et al., 2006).

На сьогодні біомеханічний вплив зовнішніх факторів на м'язове скорочення через складність методів аналізу отриманих механограм у більшості випадків здійснюється фіксацією максимальної силової відповіді. Однак, розвиток різних м’язових патологій на різних стадіях може призводити до зниження рівня утримання максимальної досягнутої силової позиції у процесі скорочення (за сталої миттєвої максимальної сили), збільшення часу досягнення максимальної сили, появи флуктуаційних складових на фазах утримання досягнутої цільової позиції, неспроможності утримувати досягнутий рівень скорочення упродовж усього періоду стимуляції, збільшення часу відновлення початкового рівня, часової розбіжності за розвитку втоми і зміни часу досягнення гладкого тетанусу [Yamasaki, et al., 2008; Kambara, et al., 2018]. Усе це може відбуватися за однакових значень максимальної сили скорочення. Відтак, пошук адекватних біомеханічних параметрів

характеризування розвитку м’язової патології хоча є складним, але необхідним завданням.

Літературні дані свідчать, що вільні радикали є одними з основних чинників у процесі пошкодження м'язової тканини. Вони ініціюють перекисне окислення ліпідів (ПОЛ), викликають пряме інгібування мітохондріальних ферментів дихального ланцюга та АТФ-азної активності, інактивацію мембранних каналів. Передбачається, що біосумісні, біодоступні та біоактивні вуглецеві наночастинки, C_{60} фулерени, можна розглядати як потужні поглиначі вільних радикалів, індукованих пошкодженнями м’язової системи. Саме підвійні хімічні зв'язки (C=C) у структурі C_{60} фулерену є електрон-дефіцитними, що й зумовлює його здатність приєднувати до 6 електронів одночасно [Goodarzi, et al., 2017; Ferreira, et al., 2018]. Усі ці дані стимулювали нас провести тестування водорозчинних C_{60} фулеренів як потенційних фармакологічних агентів, що зменшують рівень патологічних ефектів у м'язовій системі щурів. Таким чином, використання для аналізу патологічних процесів у скелетних м’язах запропонованих біомеханічних маркерів амплітудно-швидкісних змін силової відповіді та виявлення кількісних відмінностей між ними як при збільшенні ступеня тяжкості патології, так і застосуванні водорозчинних C_{60} фулеренів, представлятиме значний фундаментальний і практичний інтереси. Так, виявлений часовий алгоритм спрацювання цих біомеханічних маркерів (у певному хронологічному порядку) надає можливість використовувати його для визначення рівня м’язового пошкодження при контролі ефективності терапевтичних і реабілітаційних процедур. Водночас, виявлення позитивних ефектів водорозчинних C_{60} фулеренів на розвиток патологічних процесів у м’язовій системі відкриває перспективу їх клінічних випробувань.

Зв’язок роботи з науковими програмами, планами, темами. Дисертаційну роботу виконано в межах науково-дослідної тематики кафедри біофізики та медичної інформатики “Дослідження впливу біологічно-активних речовин різної концентрації та їх комплексів з металами на динаміку м’язового скорочення пучків волокон скелетного м’яза під впливом стимуляції електричними імпульсами” (0106U005751), проєктів науково-технічної (експериментальної) розробки МОН України «Розробка

технології застосування водорозчинних C_{60} фулеренів для зменшення втоми скелетних м'язів» (0121U109986), фундаментального дослідження МОН України «Транспорт іонів та оксалатів як ключовий фактор хронічної хвороби нирок та асоційованих з нею патологій» (0122U001535) та 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» (0123U103585).

Мета та завдання дослідження: метою роботи було проаналізувати механокінетику скорочення скелетних м'язів за експериментальних патологій та дії вуглецевих наночастинок - C_{60} фулеренів.

Для досягнення цієї мети були сформульовані наступні завдання:

1. дослідити зміни механокінетичних параметрів скорочення м'язів та біохімічних показників крові щурів за розвитку найбільш поширених патологій скелетно-м'язової системи різного ступеня тяжкості, зокрема ішемії, механічної травми, втоми та атрофії;
2. дослідити зміни механокінетичних параметрів скорочення м'язів та біохімічних показників крові щурів за різних патологій і дії водорозчинних C_{60} фулеренів у різних часових і дозових діапазонах залежно від способу введення (внутрішньом'язове та пероральне) і схеми застосування (до та після ініціації м'язової патології);
3. проаналізувати кореляцію між біомеханічними відповідями скорочення м'язів та біохімічними показниками крові щурів з метою використання механокінетичних параметрів як потенційних маркерів розвитку патологічних процесів різного ступеня тяжкості;
4. проаналізувати кореляцію між біомеханічними відповідями скорочення м'язів та біохімічними показниками крові щурів за різних патологій і дії водорозчинних C_{60} фулеренів з метою оцінки рівня м'язового пошкодження при контролі ефективності терапевтичних і реабілітаційних процедур.

Об'єкт дослідження – механокінетика скорочення скелетних м'язів.

Предмет дослідження - особливості зміни механокінетичних параметрів скорочення м’язів за розвитку найбільш поширених патологій скелетно-м’язової системи різного ступеня тяжкості та дії водорозчинних C_{60} фулеренів у різних часових і дозових діапазонах залежно від способу введення і схеми застосування.

Методи дослідження - моделювання м’язових патологій; тензометричний аналіз механокінетики скорочення скелетних м’язів; світлова та атомно-силова мікроскопії; динамічне розсіювання світла; гістологічний аналіз; біохімічний аналіз; статистичний аналіз.

Наукова новизна одержаних результатів. Результати дослідження поглиблюють фундаментальні знання щодо розуміння швидкоплинного і складного процесу скорочення скелетного м’яза на тлі розвитку найбільш поширених патологій скелетно-м’язової системи (ішемії, механічної травми, втоми та атрофії). Зокрема, вперше встановлено універсальні біомеханічні маркери амплітудно-швидкісних змін силової відповіді м’яза, «спрацювання» яких у певному хронологічному порядку дає можливість визначити ступінь тяжкості м’язової патології. На тваринних моделях підтверджена потужна, порівняно з відомими антиоксидантами, дія вуглецевих наночастинок - C_{60} фулеренів, як ефективних поглиначів вільних радикалів, індукованих пошкодженнями м’язової системи, здатних зменшити ступінь тяжкості м'язових патологій як в стані їх гострого перебігу, так і хронічних наслідків.

Практичне значення одержаних результатів. Продемонстровані на експериментальних тваринах ефекти дії водорозчинних C_{60} фулеренів у різних схемах застосування, відкривають нові можливості для створення ефективних лікарських засобів на їх основі. Доведено, що зменшення ступеня тяжкості патологічного стану м’яза за дії водорозчинних C_{60} фулеренів відображає послідовність «спрацювання» встановлених біомеханічних маркерів у зворотному порядку, що відкриває можливість використовувати цей універсальний алгоритм для визначення рівня м’язового пошкодження при контролі ефективності терапевтичних і реабілітаційних процедур.

Особистий внесок здобувача. Дисертація є самостійною науковою працею, в якій висвітлені власні ідеї і розробки автора, що дозволили вирішити поставлені

завдання. Робота містить теоретичні та методичні положення і висновки, сформульовані здобувачем особисто. Використані в дисертації ідеї, положення чи гіпотези інших авторів мають відповідні посилання.

Апробація результатів дисертації. Основні результати роботи доповідалися на 4-му З'їзді Українського біофізичного товариства (2006, Донецьк), міжнародному симпозіумі "Biological Motility: Achievements and Perspectives" (2012, Пущино), XI міжрегіональній науковій конференції (2013, Луганськ), міжнародних наукових конференціях "Психофізіологічні та вісцеральні функції в нормі і патології" (2014, Київ), міжнародних наукових конференціях "Nanotechnologies and Nanomaterials" (NANO 2017, Чернівці та 2020, 2021, Львів), VIII з'їзді Українського біофізичного товариства (2019, Київ-Луцьк), міжнародній науково-практичній конференції «Challenges in science of Nowadays» (2020, Вашингтон), а також наукових семінарах ННЦ "Інститут біології та медицини" Київського національного університету імені Тараса Шевченка.

Дисертація на здобуття наукового ступеня кандидата біологічних наук «Динаміка формування моторних команд у сенсомоторній корі котів при виконанні цілеспрямованих рухів» була захищена 27 жовтня 2003р. за спеціальністю 03.00.02 - біофізика. Матеріали і висновки кандидатської дисертації не використані в жодній частині докторської дисертації автора.

Апробація матеріалів дисертації. За матеріалами дисертації опубліковано 3 статті у наукових фахових виданнях України, 27 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, 1 монографія у міжнародному виданні, 1 патент України на винахід та 4 патенти України на корисну модель, а також 11 тез доповідей на міжнародних конференціях.

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. Протоколи дослідів було погоджено Комісією з біоетики ННЦ "Інститут біології та медицини" Київського національного університету імені Тараса Шевченка (протокол № 2 від 02 вересня 2022 року).

В експериментах використовували щурів лінії Wistar віком 1-3 місяці, вагою 110-190 г (n=7 у кожній експериментальній групі), яких утримували за стандартних умов. Для реєстрації електрофізіологічних сигналів використовували 12-ти розрядний аналого-цифровий та цифро-аналоговий перетворювач (АЦП-ЦАП). Вихідні імпульси ЦАП (DS2A, Digitimer) здійснювали стимуляцію нервів. Вхідні сигнали, через підсилювач (“Brownlee”), подавали на АЦП і реєстрували з частотою 10 кГц. Лінійний двигун у положенні сервокерування використовували для натягування м'яза та вимірювання зусилля, що розвивалося ним. Зусилля вимірювали за допомогою напівпровідникових тензодатчиків, наклеєних на жорсткі сталеві балки, встановлені на рухомі частини лінійного двигуна. Жорсткість знімача перевищувала 0,06 Н/мм, водночас як стала часу перехідних процесів довжини не перевищувала 60 мс. За попередньої підготовки до експерименту анестезію піддослідним тваринам здійснювали внутрішньочеревним введенням нембуталу (40 мг/кг). 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 відповідних 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 м’яз (*muscle soleus*) здійснювали за допомогою системи механостимуляторів. Для формування стимулюючих сигналів використовували програмовані генератори сигналів спеціальної форми.

М’язову травму викликали, стискаючи м’яз упродовж 1, 2 і 3 хв (1, 2 і 3 ступінь тяжкості травми) зажимом під тиском 3,5 кг на см^2 [Souza, et al., 2013]. Застосований crush syndrome (CS) призводив до системного прояву патологічних змін за руйнування м'язових клітин, зокрема вивільнення компонентів м'язової клітини (креатинкіназа, молочна кислота, міоглобін) у позаклітинне середовище, що слугувало маркером рівня м'язової травми. Втому викликали послідовними стимуляційними імпульсами частотою 50 Гц, тривалістю 5-6 с кожний без релаксаційного періоду між ними. Сукупність таких стимуляційних подразнень складала 500 с, після яких проводили 5-ти хв релаксацію. Кількість стимуляційних пулів дорівнювала трьом. Для ішемізації м’яза лігатурами перетягували гілку стегнової артерії, яка забезпечує його кровопостачання. Тривалість ішемізації складала 1, 2 і 3 год (1,2 і 3 ступінь тяжкості ішемії). Для моделювання знерухомлення м’яза тварин їх піддавали ахіллотенотомії – перерізу ахіллесового сухожилку [Hodgson, et al., 2013]. Досліджували групи тварин на 15, 30 і 45 добу після ахіллотенотомії.

При аналізі міотичної відповіді досліджуваного м'яза за застосування модульованого стимулюючого сигналу аналізували кілька основних біомеханічних параметрів (рис. 1), як маркерів наявності дисфункцій певної ланки у ланцюзі «збудження-відповідь», а саме:

1. зміну рівня мінімальної сили скорочення (F_{min}). За виконання досить простих односуглобових рухів цей маркер є основним показником м'язової дисфункції, феноменологічний аналіз якого дає можливість встановити наявність причинно-наслідкових зв'язків між рівнями зниження біомеханічної активності м'язів та розвитком патологічного процесу;

2. зміну часу досягнення максимальної силової відповіді (t_{max}). Зміна цього показника вказує на рівень фізіологічної дисфункції м'язового препарату під час реалізації ним максимальних силових завдань;

3. зміну рівня максимальної сили скорочення (F_{max}). Цей маркер є показником загальної дисфункції м'язової системи, що свідчить про зниження (за розвитку патологій) максимально можливої силової відповіді;

4. зміну імпульса м’язової сили. Імпульс м’язової сили (розрахована площа під силовою кривою: $S = \int_{t_1}^{t_2} F(t)dt$) є показником загальної працездатності м’яза за застосованих стимуляційних пулів;

5. зміну часу зменшення сили скорочення м'яза на 50% від початкового рівня (t_{50}) як оцінка розвитку втоми м'яза за стимуляційних подразнень;

6. зміну часу початку силової відповіді м'яза (зміна латентного періоду) (t_{start}). Цей показник дає можливість оцінити рівень патології на стадіях взаємодії м'язових міофіламентів, дисфункції кальцієвого насосу і системи саркоплазматичного ретикулуму. Ці патологічні зміни є наслідком порушення мембран міоцитів і, як правило, є показниками тяжких форм міопатії;

7. зміну часу виходу силових параметрів на початковий рівень(t_0). Збільшення внутрішньом'язових колагенових структур, наявність м'язових волокон, що не функціонують, запальні процеси знижують рівень динамічних параметрів скорочення. Досліджуваний параметр описує зміну жорсткості м'яза, пов'язаної як зі збільшенням сполучно-тканинних компонентів (тривала патологія), так і зміною внутрішньом'язового тиску (гострий період патології);

Для отримання водного колоїдного розчину C_{60} фулеренів (C_{60}FAS) був застосований метод, який ґрунтується на переведенні молекул C_{60} фулерену з толуолу у воду з подальшим обробленням ультразвуком [Turov, et al., 2010; Ritter, et al., 2015]. Одержаний C_{60}FAS за максимальної концентрації 0,15 мг/мл був стабільним упродовж 12-18 місяців за температури +4°C.

Внутрішньом’язові ін’єкції (або пероральне введення) C_{60}FAS були використані у дозах 0,5, 1 і 2 мг/кг ваги тварини за різної тривалості (1-45 діб залежно від патології) у різних схемах введення: за 1 год до ініціації патології та після ініціації патології. Також була проаналізована залишкова дія C_{60}FAS на 1 і 2 добу після припинення ін’єкцій за відповідної патології.

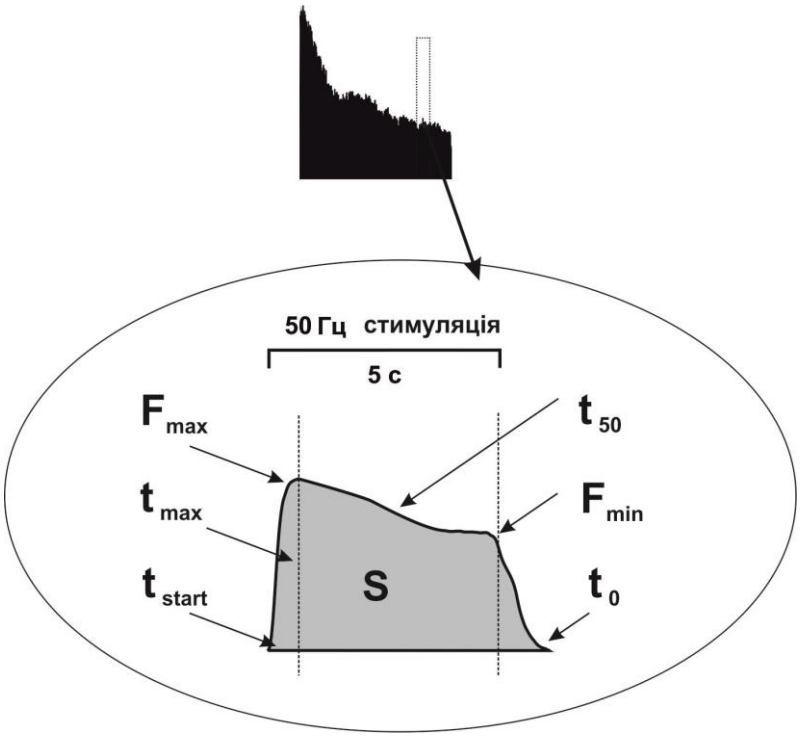

Рис. 1. Біомеханічні параметри як маркери наявності дисфункцій певної ланки міотичної відповіді у ланцюзі «збудження-відповідь»

Розмір частинок C_{60} фулерену у водному розчині визначали методами атомно-силової мікроскопії (АСМ) та динамічного розсіювання світла (ДРС) [Ritter, et al., 2015]. АСМ дані демонструють присутність у воді як поодиноких молекул C_{60}, так і їх наноагрегатів розміром від 2 до 50 нм. Метод ДРС показав, що у водному зразку значну частину (77%) складають наночастинки діаметром від 40 до 60 нм. Значення дзета-потенціалу для C_{60}FAS складало -24,5 мВ, що свідчить про негативний заряд поверхні наночастинок у розчині та його високу стабільність, тобто низьку ступінь подальшої агрегації наночастинок з часом.

Рівень креатиніну, креатинфосфокінази (КФК), лактату, лактатдегідрогенази (ЛДГ), ТБК-активних продуктів, пероксиду водню, відновленого глутатіону та активність каталази у крові піддослідних тварин, як маркерів пошкодження м’язів, визначали за допомогою клініко-діагностичного обладнання – біохімічних аналізаторів RNL-200 та JN-1101-TR2 (Нідерданди).

Статистичну обробку результатів вимірювань проводили методами варіаційної статистики з використанням програм Origin 9.4 та Statistica 13.3. При аналізі експериментальних даних у різних групах використовували t-критерій (для однієї групи) або однофакторний дисперсійний аналіз ANOVA (для трьох груп), після чого виконували множинний порівняльний тест Даннета для порівняння всіх даних з контрольними. Багатофакторний дисперсійний аналіз ANOVA використовували залежно від таких факторів варіації, як доза, тривалість застосування та схема прийому препарату. Припущення про те, що експериментальні дані відповідають

нормальному розподілу та мають ідентичні стандартні відхилення були перевірені за допомогою тестів Шапіро-Уілка та Бартлетта, відповідно. Кожна з експериментальних силових кривих, одержаних у роботі, є результатом усереднення 10-ти аналогічних експериментів. Біохімічні дані представлені як середнє значення ± стандартна помилка середнього для кожної групи (повторюваність вимірювань складала п’ять разів). Значення $p<0,05$ вважалися значущими.

ОСНОВНІ РЕЗУЛЬТАТИ ДОСЛІДЖЕНЬ ТА ЇХ ОБГОВОРЕННЯ

РОЗДІЛ 1. *Механокінетичні параметри скорочення скелетних м’язів та біохімічні показники крові щурів за розвитку м’язової ішемії*

Ішемія – один з найпоширеніших патологічних станів, який розвивається у посмугованих скелетних м’язах через раптове припинення їхнього кровопостачання. Попри те, що оклюзія периферичних артерій - серйозна клінічна проблема і найпоширеніша причина розвитку ішемії, іншими поширеними факторами її ґенезу є артеріальний тромбоз, емболія, травматичні розриви, зовнішнє стиснення і хірургічні ускладнення. Ішемічні пошкодження тканин є наслідком біохімічних реакцій, які ініціюються за гіпоксії вже після декількох хвилин після ішемізації внаслідок недостатнього кровопостачання. При цьому за ішемії тривалістю 3 години і більше відбуваються некротичні м'язові зміни і кількість некрозу у м'язовій тканині може досягати 60%. Крім того, за ішемічної реперфузії збільшується експресія адгезивних молекул на ендотелії. Залучені у вогнище пошкодження активовані нейтрофіли додатково вивільняють вільні радикали. Останні провокують вазоконстрикцію, що є характерним проявом ішемічних пошкоджень. Основна мета при лікуванні ішемії м'язів – швидке відновлення кровотоку. Однак, така терапія призводить до нового патофізіологічного процесу – реперфузійної травми, яка здатна також спричинити суттєве пошкодження м'язової тканини. Швидке встановлення ступеня тяжкості ішемічної травми має вирішальне значення для подальшої терапії, проте нині відсутні точні діагностичні тести задля досягнення цієї мети.

Враховуючи, що початок ішемічного пошкодження м'язових тканин відбувається вже в перші секунди після реперфузії, а також виходячи з можливості рівномірного розподілу введеного препарату по тканинах, для адекватного аналізу досліджуваних біомеханічних маркерів скорочення м’яза введення C_{60}FAS здійснювали внутрішньом’язово за 1 год до його ішемізації. Після ініціації ішемічного пошкодження сила скорочення *muscle soleus*, викликана 6-ти с безрелаксаційними пулами стимуляції, знизилася до 28% від контрольних значень при першому скороченні і до 9% на десятому. Зменшення імпульса м’язової сили становило 39% на першому скороченні і 6% на десятому, відповідно. Таким чином, у групі «патологія» (ішемізований м'яз) спостерігається різке зниження силової активності м'яза вже на першому скороченні. Застосування ін'єкцій C_{60}FAS збільшило силову відповідь м'яза, яка склала за дози 0,5 мг/кг - 58%, 1 мг/кг - 75% і 2 мг/кг - 79% порівняно з групою «патологія» (рис. 2).

Після застосування C_{60}FAS силова відповідь ішемізованого м'яза не зменшувалася більш ніж на 50% від контрольних значень навіть при десятому акті скорочення. Водночас, підвищення дози C_{60}FAS з 1 до 2 мг/кг не призвело до значних ефектів (рис. 2). Таким чином, передтравмове введення C_{60}FAS у дозі 1 мг/кг знижує рівень тяжкості ішемічного пошкодження м'яза на 60-75% порівняно з групою «патологія». Зменшення дози C_{60}FAS призводить до зниження ефективності його дії, а її підвищення не призводить до суттєвого збільшення біомеханічних показників скорочення м’яза.

Реєстрація сили скорочення ішемізованого м’яза при застосуванні втомлювальної стимуляції виявила зменшення імпульса м’язової сили, що становило 35% від контрольного значення (рис.3). Внутрішньом'язові ін'єкції C_{60}FAS за оптимальної дози 1 мг/кг змінили цей показник до 67% порівняно з групою «патологія». Час зменшення силової відповіді на 50% і 25% від початкових значень

та максимальна і мінімальна сили скорочення показали його 52-58% ефект на етапах утримання максимальних силових відповідей за розвитку м’язової втоми.

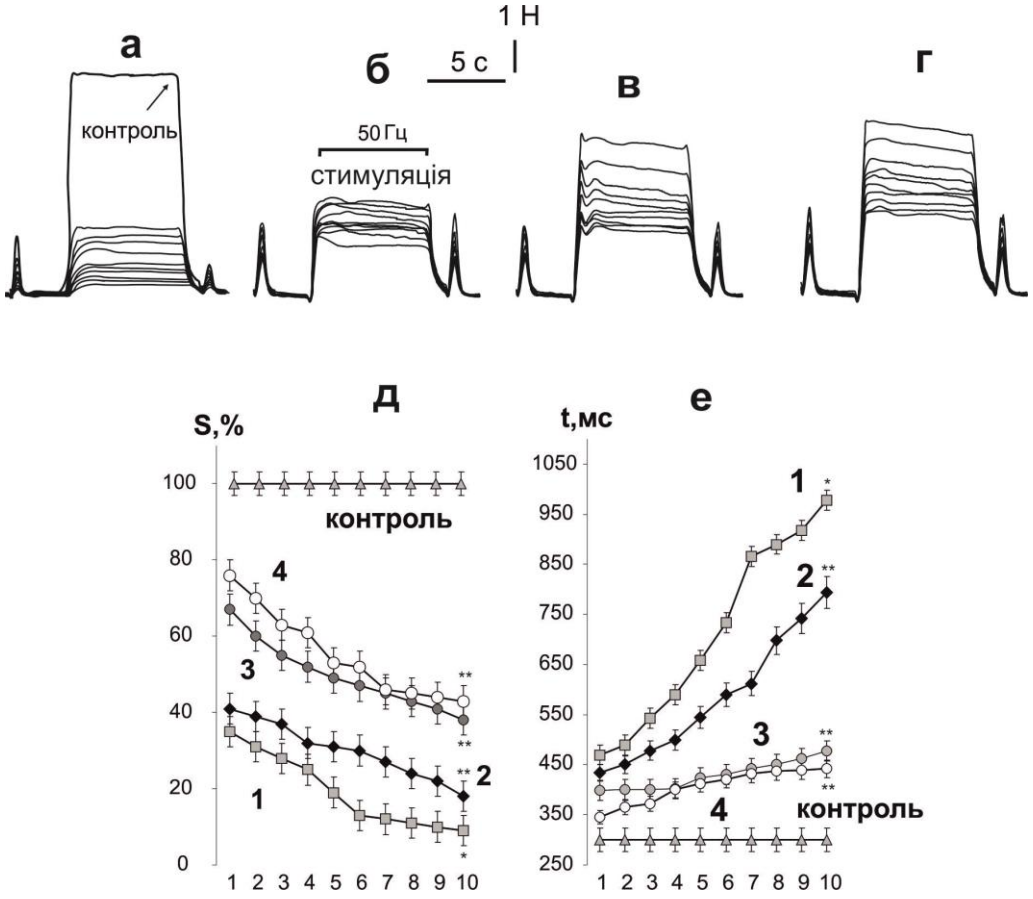

Рис. 2. Сила скорочення *muscle soleus* щура, спричинена 10-ти послідовними 6-ти с пулами стимуляції: ішемізований м'яз (контроль – нативний м'яз) (а); введення C_{60}FAS за 1 год до ішемізації м'яза у дозах: 0,5 (б), 1 (в) та 2 мг/кг (г); імпульс м’язової сили (S) у відсотках від контрольних значень (д); час досягнення максимальної силової відповіді (е). 1, 2, 3 і 4 – значення параметрів без введення C_{60}FAS та при його застосуванні у дозах 0,5, 1 і 2 мг/кг, відповідно

У процесі функціонування скелетної мускулатури найважливішим кількісним показником її роботи є швидкість виникнення гладкого тетанічного скорочення - стану безперервної напруги м'яза після виникнення повної сумації поодиноких скорочень. Навіть мінімальні зміни у структурі генерованих мотонейронами імпульсів, пошкодження мембран міоцитів, розвиток запального процесу, зміни

м'язової жорсткості, електричних властивостей мембран та тривалості гіперполяризації значно змінюють час виникнення гладких тетанічних скорочень.

Тому наступним етапом дослідження було вивчення біомеханічних маркерів виникнення гладких тетанічних скорочень. При застосуванні стимуляційних пулів наростаючої частоти ішемічно-пошкоджений м'яз упродовж усього стимуляційного пулу так і не вийшов у стадію гладкого тетанічного скорочення. Ін'єкції C_{60}FAS позитивно змінили біомеханічні параметри переходу упродовж скоротливого процесу. Крім того, важливо також зазначити, що ін'єкція C_{60}FAS усунула як стрибкоподібне зменшення сили скорочення, так і флуктуаційну складову скорочувального процесу.

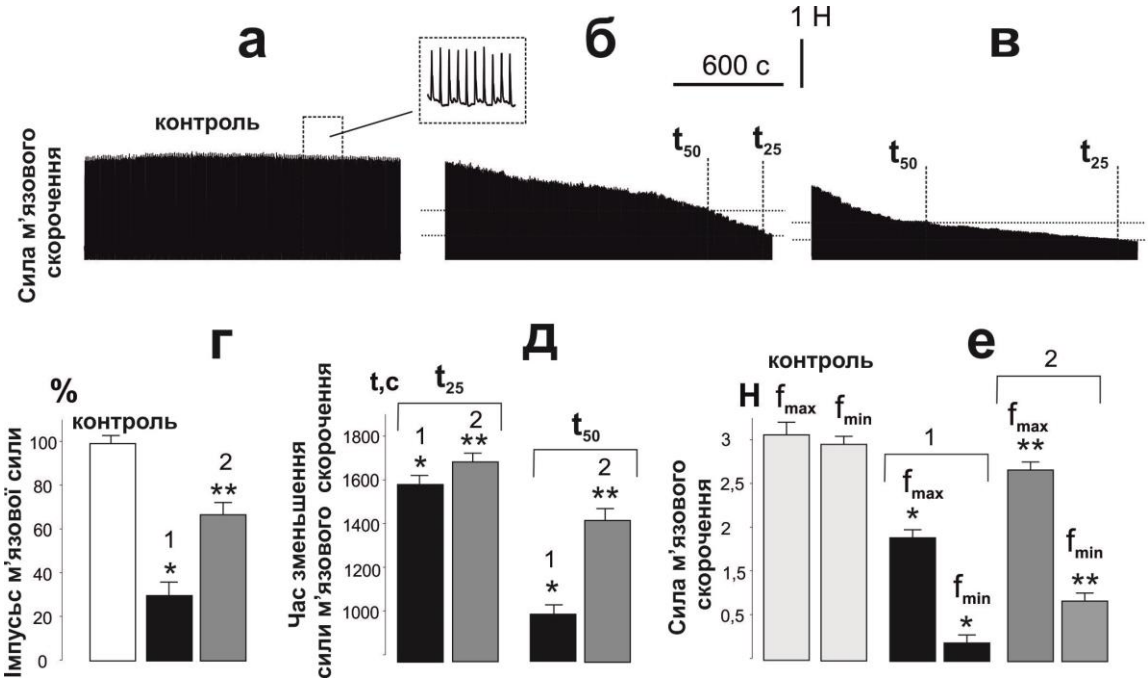

Рис. 3. Сила скорочення *muscle soleus* щура при застосуванні 1 Гц стимуляції тривалістю 1800 с: контроль - нативний м'яз (а); ішемізований м'яз (б); введення C_{60}FAS (1 мг/кг) за 1 год до ішемізації м'яза (в); імпульс м'язової сили (г); час зменшення силової відповіді на 50% та 25% від початкових значень (t_{50}, t_{25}) (д); максимальна (F_{max}) та мінімальна (F_{min}) сили скорочення м'яза (е). 1, 2 – значення параметрів без введення C_{60}FAS та при його застосуванні, відповідно. *$p<0,05$ щодо групи контроль; **$p<0,05$ щодо групи 1

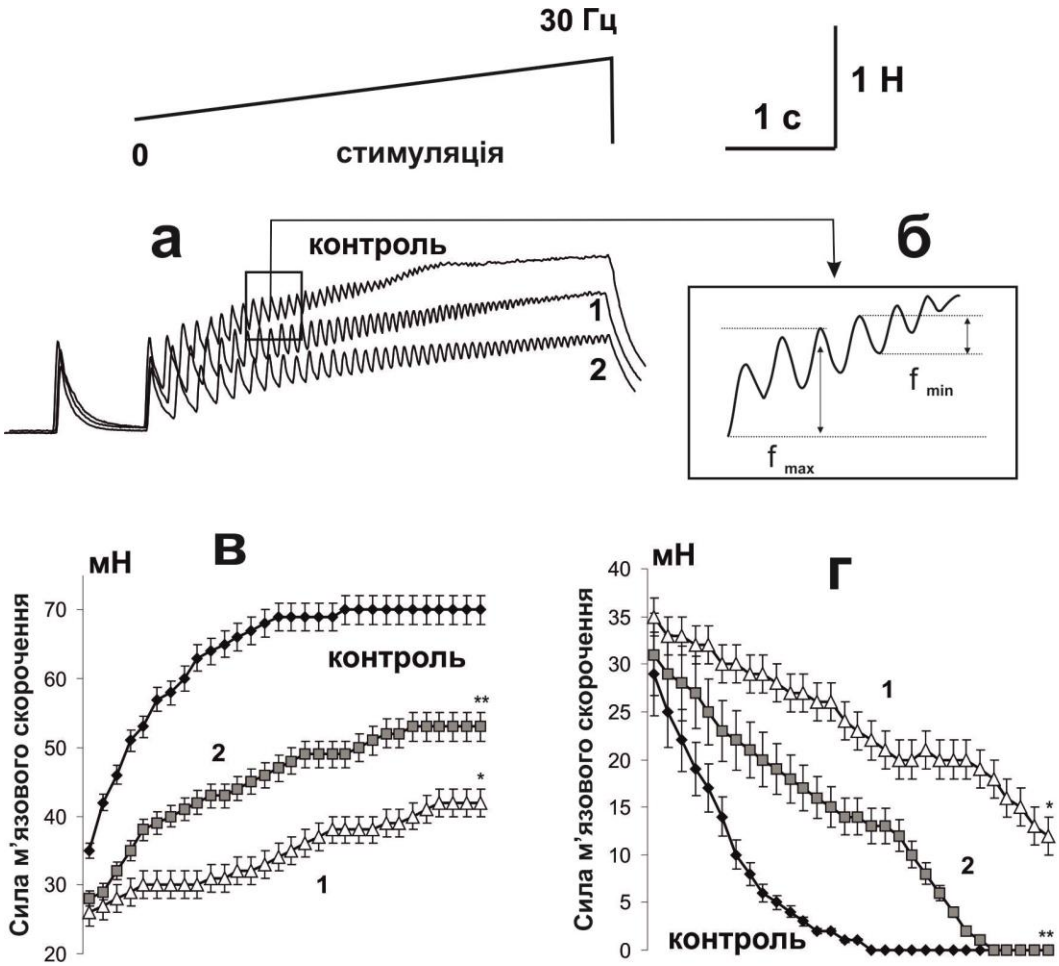

Рис. 4. Біомеханічні параметри переходу *muscle soleus* із зубчастого до гладкого тетанусу при застосуванні наростаючої стимуляції максимальної частоти 30 Гц тривалістю 6 с: механограми скорочення м'яза (а); f_{max} – максимальна сила поодинокого скорочення; f_{min} – мінімальне значення силової відповіді в одному зубці зубчастого тетанусу (б); зміни параметрів f_{max} (в) і f_{min} (г) по кожному з поодиноких скорочень до переходу силової відповіді у гладкий тетанус. 1, 2 – значення параметрів без введення C_{60}FAS та при його застосуванні (1 мг/кг), відповідно

Протекторний ефект C_{60}FAS (1 мг/кг) на біомеханічні параметри переходу ішемізованого м'яза із зубчастого у гладкий тетанус становив 68% від контрольних значень (рис. 4).

Кількісні зміни у м'язовій динаміці простежуються більш суттєво при аналізі дії ін'єкцій C_{60}FAS (1 мг/кг) на біомеханічні маркери м'язового скорочення за розвитку ішемії різного ступеня тяжкості (рис. 5). Так, зміна мінімальної сили скорочення

склала 60%, 47% і 4% у випадку 1, 2 і 3 ступеня тяжкості патології, відповідно, порівняно з групою «патологія». Позитивний ефект C_{60}FAS на цей маркер становив 65%, 56% і 28%, відповідно.

Важливо зазначити збільшення ефекту C_{60}FAS на 25-30% для перших трьох чутливих маркерів (F_{min}, t_{max} і F_{max}) зі збільшенням дози до 2 мг/кг (рис. 6). Таким чином, простежується чітка тенденція до зменшення рівня описаних біомеханічних маркерів скорочення ішемізованого м'яза зі збільшенням ступеня тяжкості патології та збільшення їх рівня за введення C_{60}FAS у дозах 1 і 2 мг/кг.

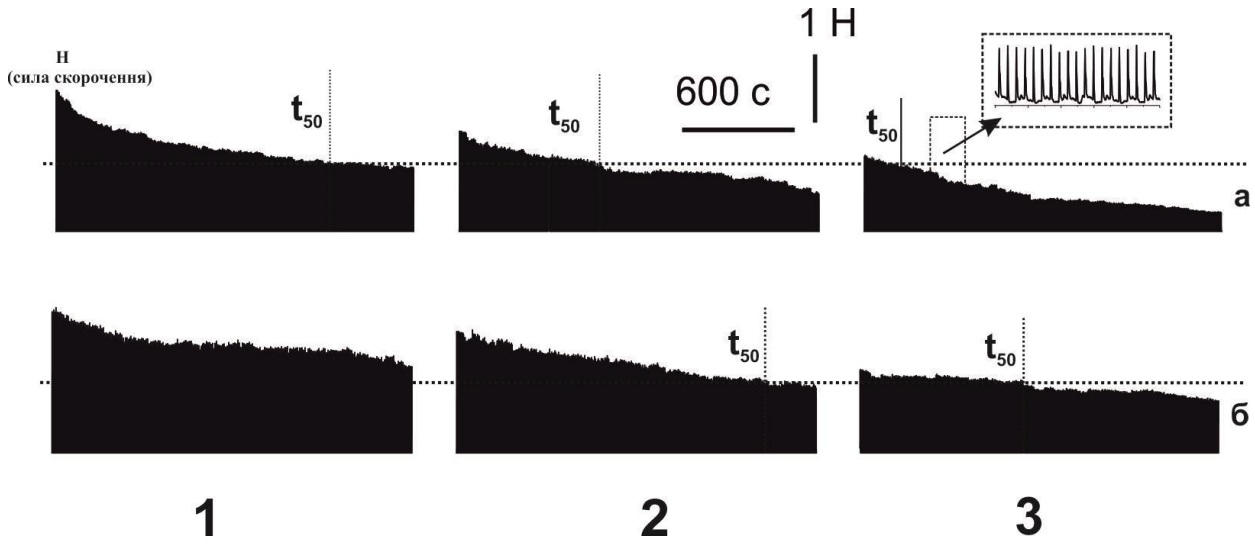

Рис. 5. Сила скорочення ішемізованого *muscle soleus* щура при застосуванні 1 Гц стимуляції тривалістю 1800 с (а) та при введенні C_{60}FAS (1 мг/кг) (б). 1, 2 і 3 – 1, 2 і 3 ступінь тяжкості патології

Аналіз біохімічних маркерів крові, які відповідають за ефективність функціонування скелетного м'яза і використовують для клінічної діагностики розвитку ішемії, зокрема рівні креатиніну, КФК, лактату та ЛДГ у крові щурів, надав можливість оцінити фізіологічні зміни у м'язі та дію препарату на патологічні процеси. Проведені дослідження показали, що усі ці маркери мають виражену тенденцію до зростання зі збільшенням ступеня тяжкості патології. Зміна рівня креатиніну – продукту, що утворюється у м'язах при руйнуванні внутрішньом'язових структур, дозволило оцінити рівень пошкодження міоцитів. За ін'єкцій C_{60}FAS у дозі

1 мг/кг рівень креатиніну знизився на 31%, 28% і 21% у випадку 1, 2 і 3 ступеня тяжкості ішемії, відповідно, порівняно з групою «патологія». Підвищення дози C_{60}FAS до 2 мг/кг додатково покращило цей показник у середньому на 10% (рис. 7).

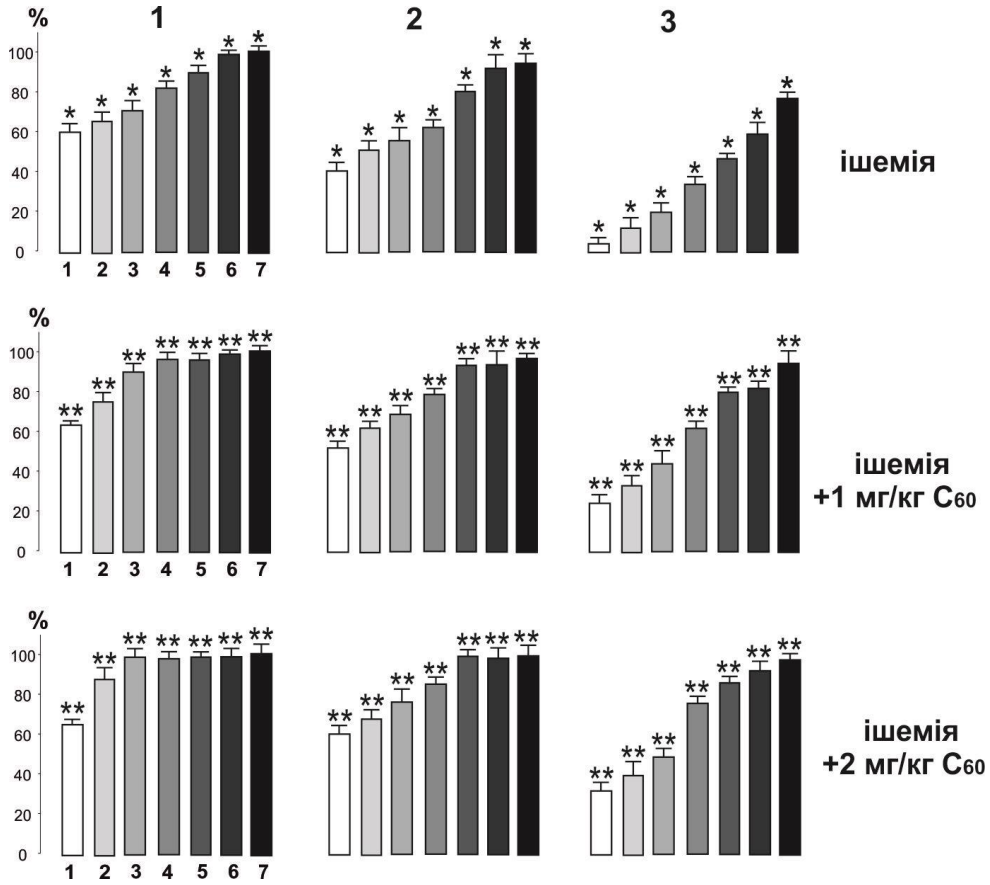

Рис. 6. Біомеханічні параметри м'язового скорочення у порядку їх максимальної зміни при аналізі м'язової ішемії: ішемія + 1 мг/кг C_{60} та ішемія + 2 мг/кг C_{60} - м'язова ішемія на тлі ін'єкцій C_{60}FAS у дозах 1 та 2 мг/кг, відповідно; 1, 2, 3 – ступінь розвитку м'язової ішемії. *p<0,05 щодо групи контроль; **p<0,05 щодо групи ішемія. 1 - F_{min} – мінімальна сила поодинокого скорочення м'яза; 2 - t_{max} - час досягнення максимальної сили скорочення м'яза; 3 - F_{max} – максимальна сила поодинокого скорочення м'яза; 4 – S – імпульс м’язової сили; 5 – t_{50} – час зменшення сили скорочення м'яза на 50% від початкового рівня; 6 – t_0 – час після припинення стимуляції до виходу м'язової сили на початкове значення; 7 - t_{start} - час між початком стимуляції та початком скорочувального процесу (затримка початку скорочення м'яза)

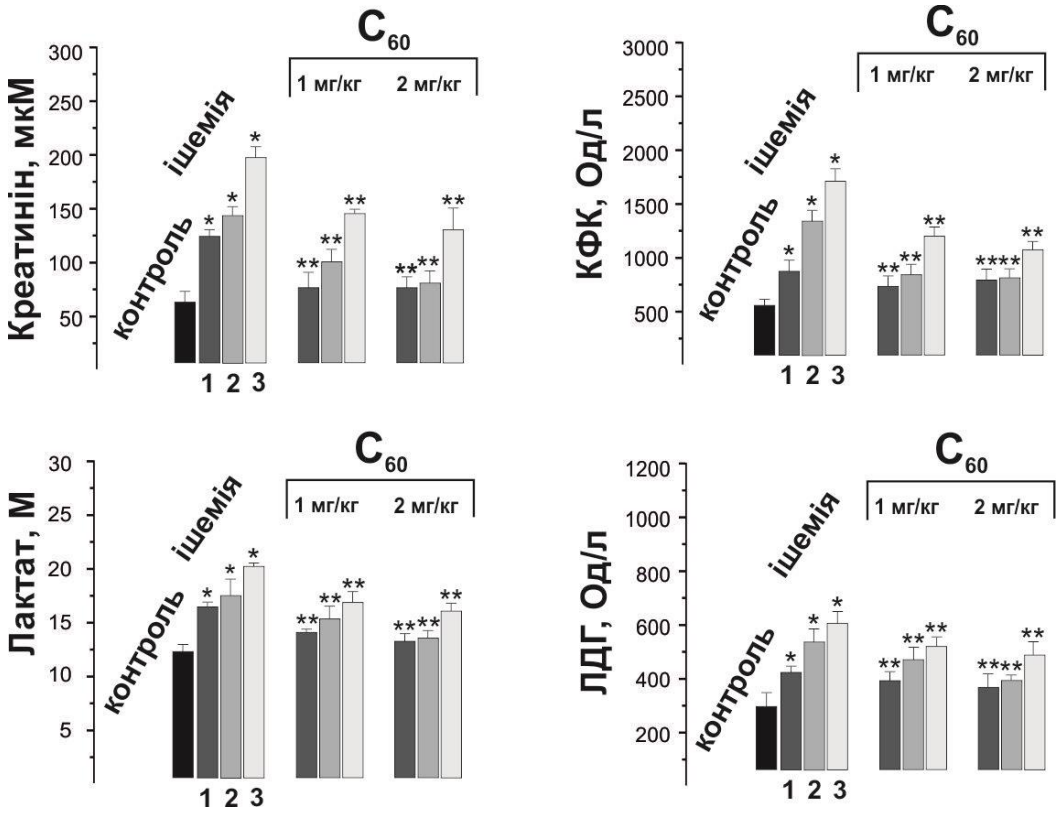

Рис. 7. Біохімічні показники розвитку патологічних процесів у *muscle soleus* щурів: ішемія - ішемізований м'яз; C_{60} - патологія на тлі ін'єкцій C_{60}FAS у дозах 1 і 2 мг/кг, відповідно; 1, 2, 3 – ступінь розвитку патологічного процесу у м'язі; *p<0,05 щодо групи контроль; **p<0,05 щодо групи ішемія

Аналіз біохімічних показників показав, що після ініціації ішемії у м’язі відбувається значне виснаження клітинних енергетичних субстанцій. Як наслідок, порушується гомеостаз та має місце втрата іонного градієнта через клітинні мембрани. Це веде до накопичення лактату та іонів H^+ і, відповідно, підкислення pH внутрішньо- та позаклітинних середовищ.

За ін'єкцій C_{60}FAS у дозі 1 мг/кг концентрація лактату знизилася на 46%, 49% та 35% у випадку 1, 2 і 3 ступеня тяжкості ішемії, відповідно, порівняно з групою «патологія». Підвищення дози C_{60}FAS до 2 мг/кг додатково покращило цей показник у середньому на 13%.

Рівень зміни ЛДГ – ферменту, який каталізує окислення молочної кислоти, дозволяє оцінити загальний стан працездатності пошкодженого м'яза після його тривалої активації. За ін'єкцій C_{60}FAS у дозі 1 мг/кг рівень ЛДГ знизився на 47%, 34%

і 26% у випадку 1, 2 і 3 ступеня тяжкості ішемії, відповідно, порівняно з групою «патологія». Підвищення дози C_{60}FAS до 2 мг/кг додатково покращило цей показник у середньому на 9%.

Одним з важливих маркерів патологічних процесів у м'язі є зміна концентрації КФК – ферменту із системи енергетичного забезпечення скелетно-м'язових клітин. При механічному пошкодженні м'язів спостерігається вихід цього ферменту з клітин та, відповідно, підвищення його активності у крові. За ін'єкцій C_{60}FAS у дозі 1 мг/кг рівень КФК знизився на 29%, 48% і 23% у випадку 1, 2 і 3 ступеня тяжкості ішемії, відповідно, порівняно з групою «патологія». Підвищення дози C_{60}FAS до 2 мг/кг додатково покращило цей показник у середньому на 8%.

Таким чином, при введенні C_{60}FAS у дозі 1 мг/кг зменшення описаних біохімічних параметрів за ішемічних пошкоджень м’яза відбувається на рівні 35% порівняно з групою «патологія». Підвищення дози C_{60}FAS до 2 мг/кг додатково покращило ці показники на 7-10%.

Отже, біохімічні показники ішемічного пошкодження м’язової тканини корелюють з описаними вище біомеханічними параметрами як зі збільшенням ступеня тяжкості пошкодження, так і за протекторної дії C_{60}FAS.

Патологічні процеси, що виникають відразу після ініціації ішемічного пошкодження, є джерелом активних форм кисню (АФК) та сприяють інтенсифікації ПОЛ. Для підтвердження припущення позитивного ефекту ін’єкцій C_{60}FAS шляхом модифікації АФК-залежних механізмів та його впливу на активність ендогенних антиоксидантів ми проаналізували показники про- та антиоксидантного балансу (каталази, перекису водню, ТБК-активних продуктів та відновленого глутатіону) у крові щурів після ініціації м’язової ішемії (рис. 8).

За ін'єкцій C_{60}FAS у дозі 1 мг/кг активність каталази знизилася на 33%, 47% і 22% у випадку 1, 2 і 3 ступеня тяжкості ішемії порівняно з групою «патологія».

Концентрація перекису водню за ін'єкцій C_{60}FAS зменшилася на 29%, 35% і 22%, відповідно. Концентрація ТБК-активних продуктів за ін'єкцій C_{60}FAS знизилася на 33%, 38% і 27%, відповідно. Кількість відновленого глутатіону за ін'єкцій C_{60}FAS знизилася на 29%, 33% і 25%, відповідно, порівняно з групою

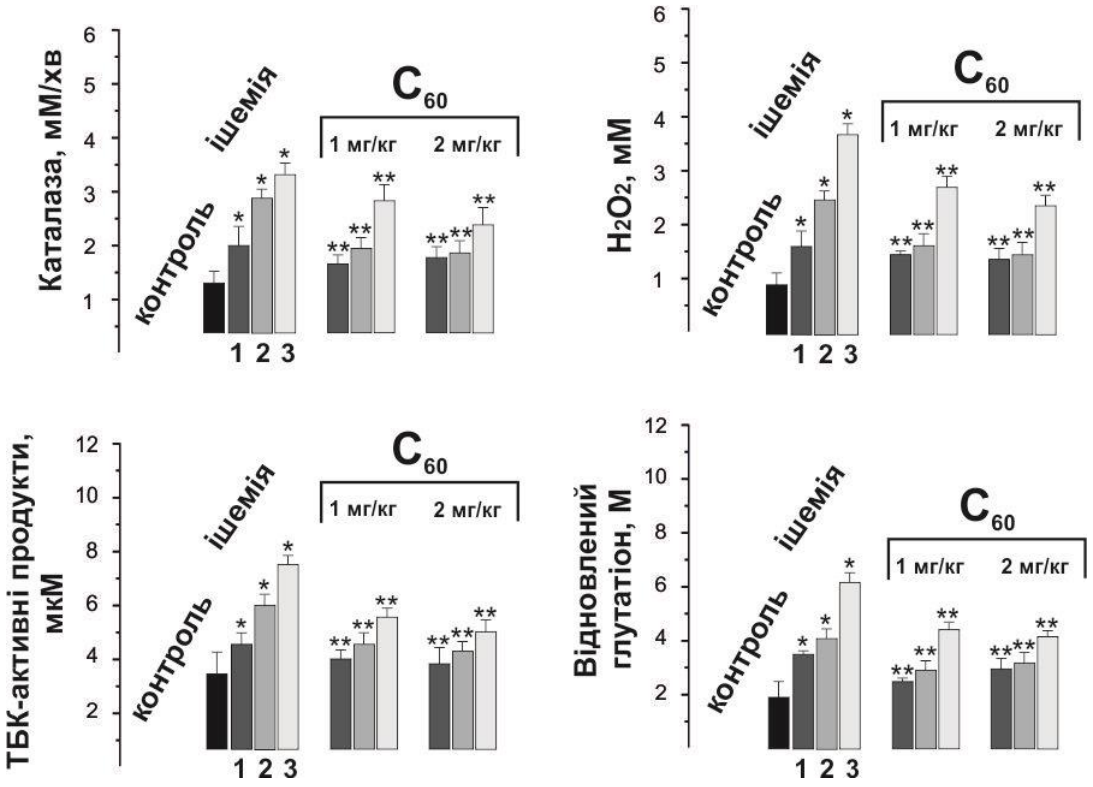

Рис. 8. Показники про- та антиоксидантного балансу в крові щурів: ішемія - ішемізований м'яз; C_{60} - патологія на тлі ін'єкцій C_{60}FAS у дозах 1 і 2 мг/кг, відповідно; 1, 2, 3 – ступінь розвитку патологічного процесу у м'язі; *p<0,05 щодо групи контроль; **p<0,05 щодо групи ішемія

«патологія». Збільшення дози C_{60}FAS до 2 мг/кг додатково покращило усі описані показники у середньому на 8-9%.

Таким чином, при аналізі різного ступеня тяжкості ішемічного пошкодження м’язів прослідковується чітка кореляція між зміною біохімічних показників розвитку патологічних процесів у м'язі, показниками про- та антиоксидантного балансу та досліджуваними біомеханічними маркерами скорочення скелетного м’яза.

При аналізі ефекту C_{60}FAS на функціонування скелетного м’яза щурів за введення препарату після ініціації патології встановлено, що найбільш оптимальним для корекції швидкісних макропараметрів скорочення ішемізованого м’яза є його внутрішньовенне введення. Водночас, внутрішньом’язове введення C_{60}FAS проявляє більш виражений вплив при рухах, пов’язаних з генерацією максимальних силових відповідей або тривалих скорочень, які збільшують рівень втоми м’яза. Аналіз

біохімічних маркерів крові, які відповідають за ефективність функціонування скелетного м'яза і використовують для клінічної діагностики розвитку ішемії засвідчив, що їх рівень суттєво знижується за оптимальної дози введення C_{60}FAS (1 мг/кг) порівняно з групою «патологія».

Таким чином, одержані результати вказують на те, що C_{60}FAS може бути перспективним засобом для профілактики і корекції ішемічно-пошкодженої функції скелетних м'язів.

Основні публікації за цим розділом:

1. **D.M. Nozdrenko**, D.O. Zavodovskyi, T.Yu. Matvienko, S.Yu. Zay, K.I. Bogutska, Yu.I. Prylutskyy, U. Ritter, P. Scharff. C_{60} Fullerene as Promising Therapeutic Agent for the Prevention and Correction of Skeletal Muscle Functioning at Ischemic Injury. Nanoscale Research Letters, 2017, 12(1): 115. **(Q2)**

2. **D. Nozdrenko**, T. Matvienko, O. Vygovska, K. Bogutska, O. Motuziuk, N. Nurishchenko, Yu. Prylutskyy, P. Scharff, U. Ritter. Protective Effect of Water-Soluble C_{60} Fullerene Nanoparticles on the Ischemia-Reperfusion Injury of the Muscle Soleus in Rats. International Journal of Molecular Sciences, 2021, 22(13): 6812. **(Q2)**

РОЗДІЛ 2. *Механокінетичні параметри скорочення скелетних м'язів та біохімічні показники крові щурів на тлі м'язової травми*

Усі механічні м'язові травми можна умовно поділити на три групи: контузія, контузія з пошкодженням судин та розрив м'язової тканини. Загалом біля 90% травм складають саме контузії, а на розрив м'язової тканини припадає лише 10%, однак саме вони викликають найскладніші наслідки. Фізіопатологія, що виникає внаслідок м'язової травми, характеризується такими явищами як розрив сарколеми та саркомерних одиниць, пошкодження мембрани, а далі - проникнення позаклітинного кальцію у м'язову клітину, що спричинює руйнування цих структур, апоптоз, розвиток запального процесу та некроз м'язової тканини. Слід зауважити, що пошкодження груп м'язів може мати нерівномірний характер, що негативно впливає

на ідентифікацію ступеня тяжкості пошкодження. Таким чином, критично важливою є адекватна класифікація м'язових травм, необхідна для розуміння рівня пошкодження та оптимізації процесу його лікування.

Після ініціація м'язової травми спостерігається значне зменшення силової реакції м'яза з прогресуючою симптоматикою. Так, виявлено різке зниження силової відповіді м'яза вже на перших секундах стимуляції з подальшим зниженням до 43%, 38% і 19% щодо контрольних значень у випадку 1, 2 і 3 ступеня тяжкості м'язової травми, відповідно.

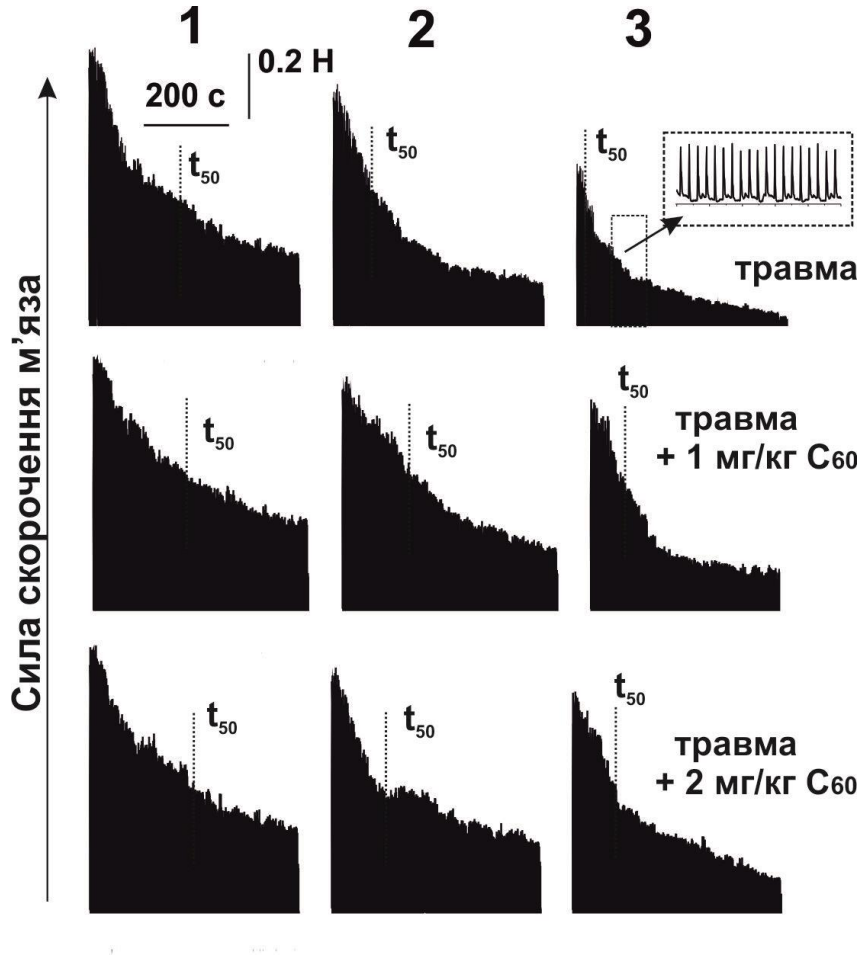

Рис. 9. Сила скорочення *muscle soleus* щурів при застосуванні 50 Гц стимуляції загальною тривалістю 600 с без релаксації: травма – м'язова травма; травма+1 мг/кг C_{60} та травма+2 мг/кг C_{60} - патологія на тлі ін'єкцій C_{60}FAS у дозах 1 та 2 мг/кг, відповідно; 1, 2, 3 – ступінь розвитку патологічного процесу у м'язі

Застосування внутрішньом’язових ін'єкцій C_{60}FAS у дозі 1 мг/кг за 1 год до ініціації травми збільшило силову відповідь м'яза на 21%, 19% і 12% у випадку 1, 2 і 3 ступеня тяжкості м’язової травми, відповідно, порівняно з групою «патологія» (рис. 9). Підвищення дози C_{60}FAS до 2 мг/кг додатково збільшило силову відповідь на 8%, 9% і 7%, відповідно. За використання втомлювальної стимуляції максимальні силові відповіді травмованого *muscle soleus* зменшувалися на 51%, 73% і 88% у випадку 1, 2 і 3 ступеня тяжкості м'язової травми, відповідно, та на 12%, 23% і 31% на 1, 2 і 3 добу після м'язової травми, відповідно. Застосування ін'єкцій C_{60}FASза оптимальної дози 1 мг/кг збільшило максимальні силові відповіді на 11%, 14% і 17% у випадку 1, 2 і 3 ступеня тяжкості м'язової травми, відповідно, порівняно з групою «патологія» (рис. 10).

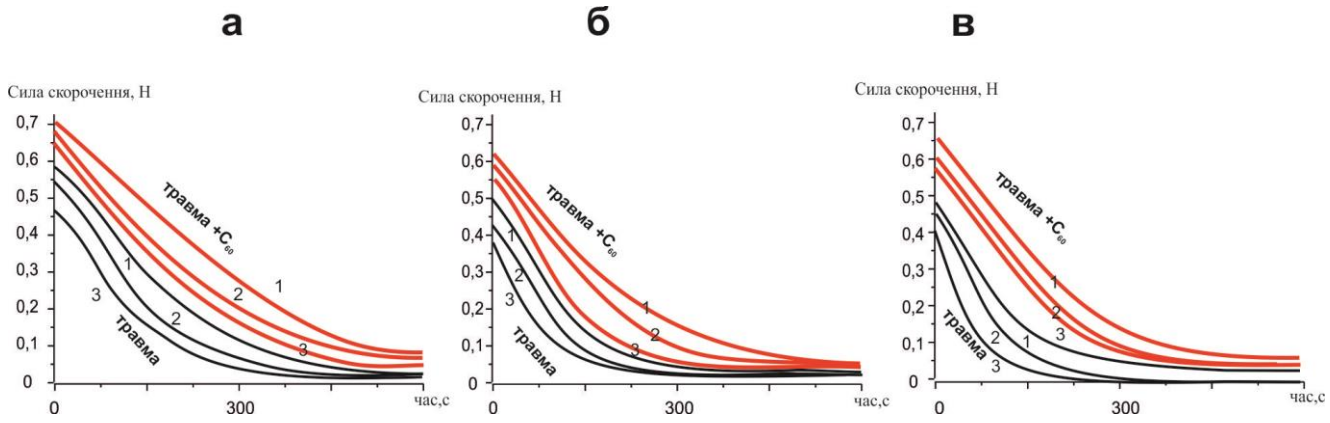

Рис. 10. Криві максимальних силових відповідей травмованого *muscle soleus*, викликані стимуляційним сигналом частою 1 Гц і тривалістю 1800 с, при застосуванні ін'єкцій C_{60}FAS (1 мг/кг): травма, травма+C_{60} - травма, травма на тлі ін'єкцій C_{60}FAS; 1, 2, 3 - криві максимальних силових відповідей за розвитку 1, 2, 3 ступеня патологічного процесу у м'язі; а, б, в - криві максимальних силових відповідей на 1, 2 і 3 добу після м'язової травми

Відносно незначний ефект C_{60}FAS можна пояснити тим, що м'язова травма з розривом м'язових тканин є тяжкою патологією, що охоплює не лише м'язові структури, але й ускладнена больовою симптоматикою високого рівня. Тому застосування C_{60}FAS, на нашу думку, не є достатнім для подолання такої м’язової

патології. Використання для аналізу досліджуваних патологічних процесів біомеханічних маркерів амплітудно-швидкісних змін силової відповіді виявило суттєві кількісні відмінності між ними за протекторної дії C_{60}FAS. Так зміна мінімальної сили скорочення при м'язовій травмі склала 31%, 17% і 4% щодо норми у випадку 1, 2 та 3 ступеня тяжкості патології, відповідно (рис. 11).

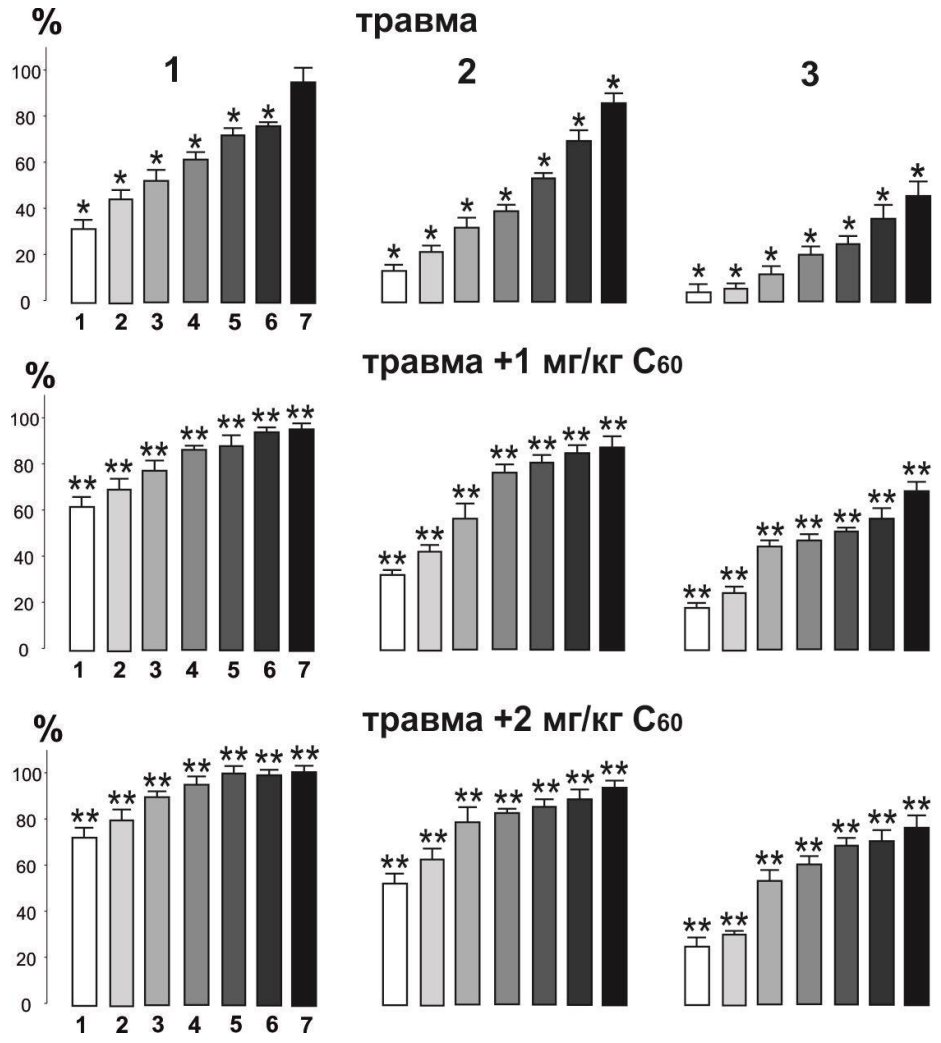

Рис. 11. Біомеханічні параметри в порядку їх максимальної зміни за м'язової травми: 1, 2, 3 – ступінь тяжкості травми; 1 - F_{min}- мінімальна сила скорочення м'яза; 2 - t_{max} - час досягнення максимальної сили скорочення м'яза; 3 - F_{max} - максимальна сила скорочення м'яза; 4 – S - імпульс м'язової сили; 5 - t_{50} - час зменшення сили м'яза на 50% від початкового рівня; 6 - t_0 - час після припинення стимуляції до виходу м'язової сили на початкове значення; 7 - t_{start} - час між початком стимуляції та початком скорочення

Позитивний ефект C_{60}FAS (1 мг/кг) на цей маркер становив 60%, 23% і 18%, відповідно, порівняно з групою «патологія». Зміна часу досягнення максимальної сили скорочення м'яза при м'язовій травмі склала 42%, 21% і 6% щодо норми у випадку 1, 2 та 3 ступеня тяжкості патології, відповідно. Позитивний ефект C_{60}FAS(1 мг/кг) на цей маркер становив 67%, 38% і 21%, відповідно, порівняно з групою «патологія». Маркери F_{max}, S і t_{50} виявили таку ж тенденцію до змін за протекторної дії C_{60}FAS (1 мг/кг) у випадку 1, 2 і 3 ступеня тяжкості м'язової травми. Водночас, маркери t_0 і t_{start} змінювалися лише у випадку 2 і 3 ступеня розвитку патологічного процесу (76% і 49% щодо норми) та відновлювалися на 92–95% при застосуванні ін'єкцій C_{60}FAS (1 мг/кг).При підвищенні дози C_{60}FAS до 2 мг/кг усі досліджувані маркери додатково зростали: від 35% для найбільш чутливого маркера F_{min} до 10% для найменш чутливого маркера t_{start}.

Проведені дослідження показали, що усі біохімічні маркери, які відповідають за ефективність функціонування скелетного м'яза та використовуються для діагностики рівнів ушкодження м’язів, мають виражену тенденцію до зростання зі збільшенням ступеня тяжкості травми та часу після її ініціації, що свідчить про виконання м'язової системою надмірної для її фізіологічного рівня роботи з подальшим прогресуючим розвитком м'язової втоми. Так, рівень креатиніну збільшився до 153%, 217% і 283% у випадку 1, 2 і 3 ступеня тяжкості м'язової травми, відповідно (рис. 12). За ін'єкцій C_{60}FAS у дозі 1 мг/кг концентрація креатині ну знизилася на 9%, 6% і 7%, відповідно, порівняно з групою «патологія». Підвищення дози C_{60}FAS до 2 мг/кг додатково покращило цей показник не більше ніж на 3%.

Збільшення фракції КФК на 142%, 179% і 243%, відповідно, є результатом руйнувань стінок міоцитів з частковим виходом внутрішньоміоцитних ферментів в екстрацелюлярний простір. За ін'єкцій C_{60}FAS у дозі 1 мг/кг її рівень знизився на 9%, 8% і 10% у випадку 1, 2 і 3 ступеня тяжкості м'язової травми, відповідно,порівняно з групою «патологія» та додатково знижувався ще на 5-6% при підвищенні дози C_{60}FAS до 2 мг/кг.

За ін'єкцій C_{60}FAS у дозі 1 мг/кг концентрація лактату знизилася на 11%, 9% і 10% у випадку 1, 2 і 3 ступеня тяжкості м'язової травми, відповідно, порівняно з

групою «патологія» та додатково знижувалася ще на 3% при підвищенні дози C_{60}FAS до 2 мг/кг.

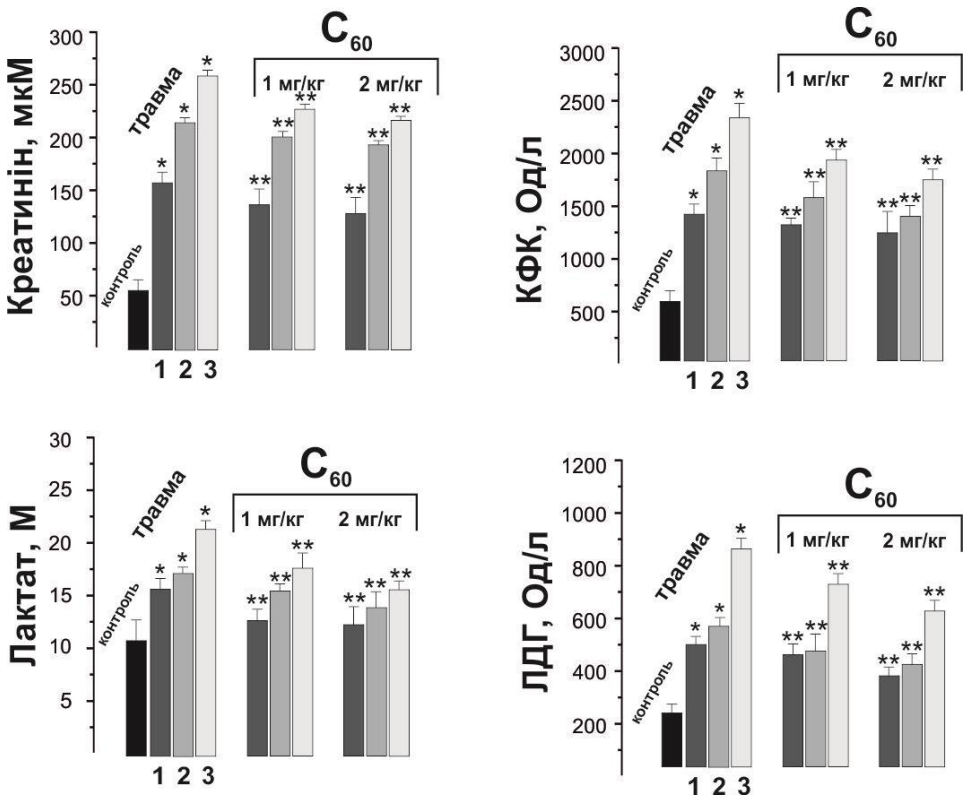

Рис. 12. Біохімічні показники розвитку патологічних процесів у *muscle soleus* щурів: травма - травмований м'яз; C_{60} - патологія на тлі ін'єкцій C_{60}FAS у дозах 1 та 2 мг/кг, відповідно; 1, 2, 3 – ступінь розвитку патологічного процесу у м'язі; *p<0,05 щодо групи контроль; **p<0,05 щодо групи травма

Зміна рівня ЛДГ на 178%, 221% і 387% у випадку 1, 2 і 3 ступеня тяжкості м'язової травми, відповідно, свідчить про розвиток суттєвих дисфункцій м'яза, пов'язаних з надлишком втомних сполук. За ін'єкцій C_{60}FAS у дозі 1 мг/кг її рівень знизився на 7%, 9% і 6%, відповідно, порівняно з групою «патологія» та додатково знижувався ще на 4% при підвищенні дози C_{60}FAS до 2 мг/кг.

Таким чином, біохімічні маркери розвитку досліджуваної патології показують позитивний ефект ін’єкцій C_{60}FAS на рівні 12-15%. Водночас, біомеханічні маркери описують позитивний ефект C_{60}FAS на рівні 35-60%. Це, на нашу думку, є результатом негативного впливу на біохімічні маркери супутніх патологічних процесів, пов’язаних з розвитком запалення, та більш ефективного впливу ін’єкцій

C_{60}FAS на найбільш уразливі фази скоротливого процесу, відповідальні за точністне позиціонування суглобу.

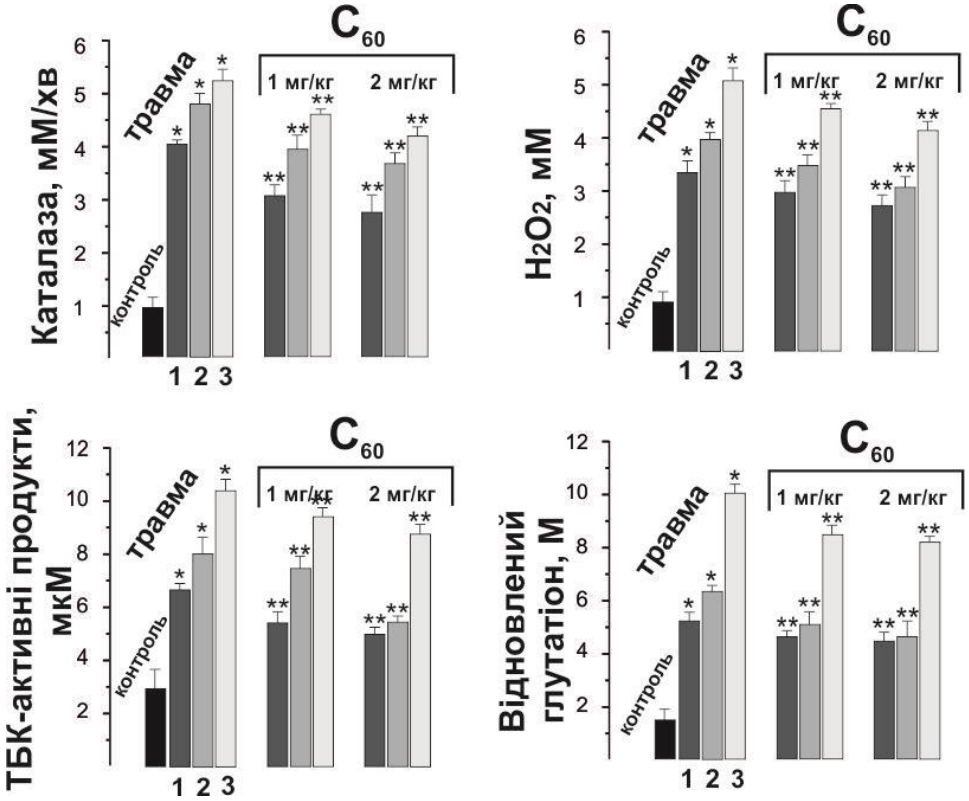

Рис. 13. Показники про- та антиоксидантного балансу у крові щурів: травма - травмований м'яз; C_{60} - патологія на тлі ін'єкцій C_{60}FAS у дозах 1 та 2 мг/кг, відповідно; 1, 2, 3 – ступінь розвитку патологічного процесу у м'язі; *p<0,05 щодо групи контроль; **p<0,05 щодо групи травма

Показники про- та антиоксидантного балансу (активності каталази, концентрації перекису водню, ТБК-активних продуктів та відновленого глутатіону) у крові щурів достовірно корелювали з рівнем розвитку патологічного процесу у скелетному м’язі та позитивно змінювалися за ін’єкцій C_{60}FAS (рис. 13).

Таким чином, водорозчинні C_{60} фулерени здатні впливати на активність ендогенних антиоксидантів, перешкоджаючи виникненню дисфункції у травмованому м'язі, однак адекватний аналіз цього впливу можливий лише з урахуванням відповідних біомеханічних маркерів.

Дослідження дії C_{60}FAS на цю м'язову патологію за внутрішньом’язового введення препарату після ініціації травми виявило позитивний вплив на

механокінетичні показники скорочення м'язів щурів. В електрофізіологічних та біохімічних дослідженнях показано, що застосування C_{60}FAS призводить до скорочення часу відновлення сили м'язового скорочення, збільшення часу м'язової витривалості та зниження рівня патологічних змін. На підставі отриманих даних можна припустити, що антиоксидантна дія водорозчинних C_{60} фулеренів впливає на розвиток запального процесу в пошкодженому м'язі: запальні реакції є наслідком генерування у пошкоджених тканинах великої кількості вільнорадикальних агентів, які, насамперед, здатні руйнувати мембранні клітинні оболонки. Збільшення біомеханічних показників функціонування м’яза пов'язане з інактивацією C_{60} фулеренами надлишкової кількості продуктів вторинного окислення у волокнах.

Основні публікації за цим розділом:

1. **D. Nozdrenko**, T. Matvienko, O. Vygovska, V. Soroca, K. Bogutska, A. Zholos, P. Scharff, U. Ritter, Yu. Prylutskyy. Post-traumatic recovery of *muscle soleus* in rats is improved via synergistic effect of C_{60} fullerene and TRPM8 agonist menthol. Applied Nanoscience, 2022, 12(3), 467–478. **(Q2)**

РОЗДІЛ 3. *Фактори розвитку та шляхи корекції м’язової втоми*

У процесі формування м'язової втоми відбувається порушення метаболізму, утворюються продукти неповного окислення кисню, перекиси та вільні радикали. Надмірне накопичення АФК (оксидативний стрес) веде до серйозних функціональних порушень, оскільки пошкоджуються різні компоненти клітин. Зниження працездатності м’яза за його тривалого подразнення зумовлено двома основними причинами. Першою з них є те, що під час скорочення м’яза у ньому накопичуються продукти обміну речовин (молочна кислота, вільні радикали та ін.), частина з яких, а також іони калію, дифундують з волокон назовні та пригнічують здатність збудливої мембрани міоцитів генерувати потенціали дії. Інша причина – це поступове виснаження енергетичних запасів.

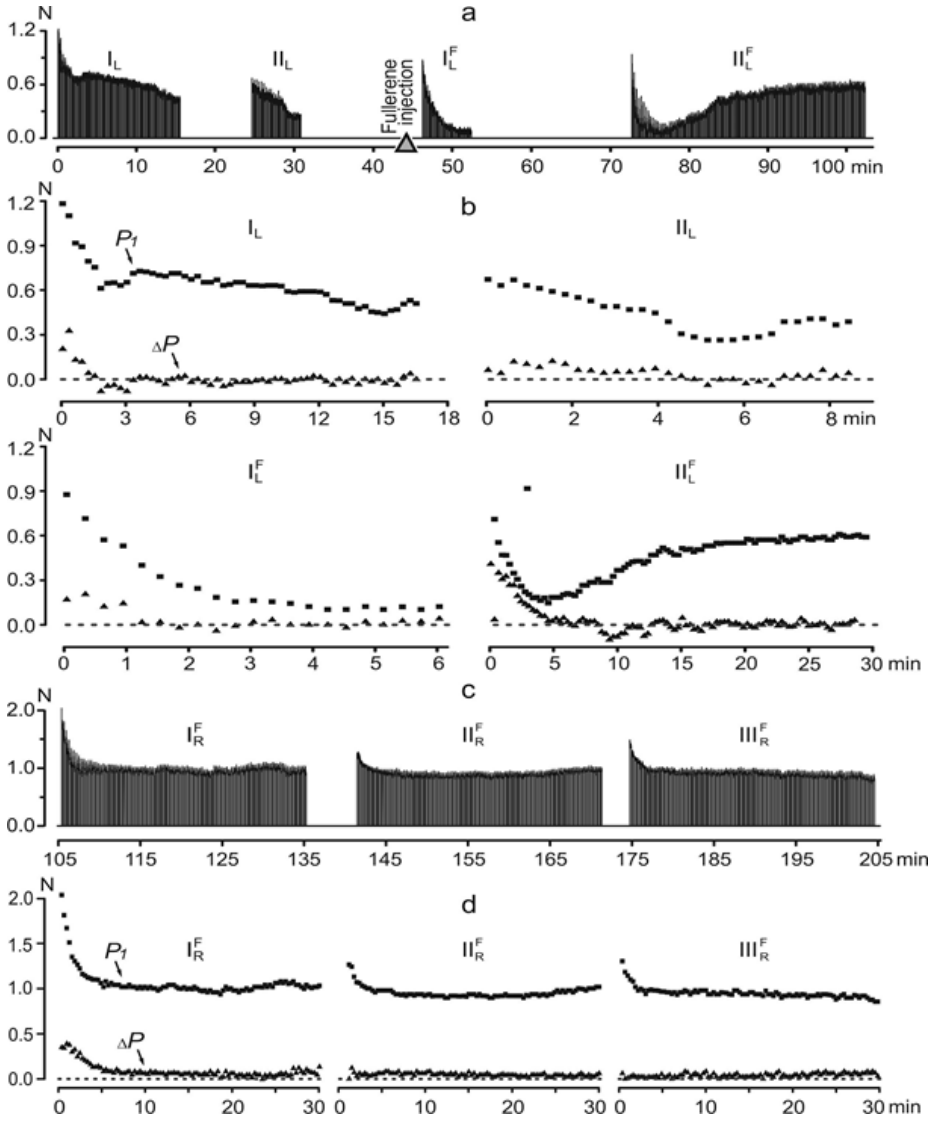

Рис. 14. Сила скорочення (Н) лівої і правої кінцівок щура з часом (хв), викликаних втомлювальною стимуляцією до та після введення C_{60}FAS (1 мг/кг) у ліву кінцівку: (а,в) – скорочення лівої і правої кінцівки, відповідно (трикутником відзначений момент ін'єкції); ,г) - амплітудні значення сили на початку поодиноких тетанічних скорочень (ΔР - різниця між значеннями сили на початку та наприкінці скорочення). Швидке формування втоми (падіння силової відповіді м'яза на понад 50%) призводило до необхідності зменшення тривалості періодів стимуляції (I–III). L і R - ліва і права кінцівки, відповідно; C_{60} - реєстрація зусилля після введення C_{60}FAS у ліву кінцівку

За тривалого функціонування м’яза відбувається різке зменшення запасів глікогену, внаслідок чого порушуються процеси ресинтезу АТФ і креатинфосфату,

що є необхідним для здійснення м’язового скорочення. Захист клітин від таких пошкоджень забезпечується антиоксидантною системою. Метою наступного етапу роботи було оцінити дію C_{60}FAS на динаміку відновлення скорочувальних властивостей скелетного м'яза щура за формування його втоми, викликаної тривалою активацією.

Внаслідок використання втомлювальної стимуляції м'яза на лівій кінцівці, 50% рівень втоми у ньому був досягнутий за 12 хв. Після 10 хв відпочинку сила тетанічних скорочень дещо відновлювалася, проте вона не досягала початкового рівня і продовжувала швидко спадати. У цьому випадку також відбувалося одночасне зниження динамічної компоненти падіння сили (рис. 14)

Після того як м'яз досяг втоми (фіксували падіння силової відповіді м'яза на понад 50%), внутрішньом'язово вводили C_{60}FAS у дозі 1 мг/кг. Ін’єкції C_{60}FAS призвели до поступового відновлення рівня ізометричного зусилля через 32 хв після введення препарату.

Через 50 хв після введення препарату сила, що розвивається м'язом, виходила на певний стаціонарний рівень, який утримувався упродовж усієї експериментальної серії. Важливо відзначити, що м'яз підтримував силовий рівень, що розвивається, ще упродовж 1 год. Загальний час зменшення сили скорочення м'яза на 50%, після введення препарату, складало 120 хв. Для порівняння, у контролі тривалість цього періоду виникнення втоми становила 42 хв. Слід відзначити, що через 50 хв, механограми лівої і правої кінцівок показували однаковий результат, що може бути доказом розповсюдження C_{60}FAS на усі м’язові групи тварини. Відновлення рівня силової відповіді активного м'яза наприкінці експериментальної серії було статистично достовірним порівняно з таким на початку і практично досягало своїх контрольних значень. Аналіз показав статистично значущий вплив таких факторів як введення препарату і часу після цього, так і їх взаємодію.

Таким чином, дія C_{60}FAS веде, з одного боку, до зменшення часу відновлення сили скорочення м'яза (після стану його повної втоми), а з іншого – збільшує у кілька разів час активного функціонування м'яза до появи суттєвих проявів його втоми.

Відомо, що у структурі м’язів розрізняють волокна двох типів: повільно та швидко скорочувальні. Ці волокна представляють собою різні функціональні одиниці, що відрізняються не лише скорочувальними, але й морфологічними та біохімічними властивостями. Динаміка накопичення продуктів ліпопероксидації і тривалість таких змін залежать від багатьох факторів, одним з яких є тип м’язових волокон, оскільки останнім притаманні специфічні метаболічні реакції та особливості антиоксидантного захисту. Відтак, на наступному етапі ми вивчали антиоксидантну дію водорозчинних C_{60} фулеренів на біомеханіку скорочення швидкого та повільного м’язів щурів за розвитку м’язової втоми.

Реєстрація сили скорочень *muscle soleus* та *muscle gastrocnemius* стимуляційними пулами виявила істотну відмінність у розвитку їх втомлювальних процесів, яка склала 31%, 39% і 47% на 1, 2 і 3 пулі стимуляції, відповідно (рис. 15). Ці результати підтверджують дані про більшу чутливість швидких м'язових волокон до розвитку втоми. Механограми, отримані через 1 годину після введення C_{60}FAS, продемонстрували виражений підвищений ефект C_{60} фулерену на *muscle gastrocnemius*, що був на 24%, 26% і 29% більшим, чим у випадку *muscle soleus* на 1, 2 і 3 пулі стимуляції, відповідно.

Аналіз значень імпульсу м’язової сили через 1 год після введення C_{60}FAS на 1,2,3,4 і 5 добу показав збільшення його компенсаторної дії. Приріст позитивного ефекту становив 8%, 6% і 4% на 5 добу у випадку *muscle soleus* і 14%, 12% і 6% - у випадку *muscle gastrocnemius* на 1, 2 і 3 пулі стимуляції, відповідно, порівняно з групою «патологія». Таким чином, отримані дані щодо силової відповіді м'яза на тлі розвитку втоми свідчать про те, що введення C_{60}FAS (упродовж мінімум 5 діб) знижує рівень тяжкості патологічних процесів на 35-45% у повільному м'язі та на 60-65% у швидкому порівняно з групою «патологія» (рис. 15).

Проведений біомеханічний аналіз маркерів розвитку втоми упродовж 2-х діб після 5-ти добового застосування C_{60}FAS показав, що після припинення ін'єкцій препарату величина імпульсу м’язової сили *muscle soleus* на першу добу незначно відрізняється від контролю (15-17%), а на другу добу – різниця з контрольними значеннями складала 5-8% (рис. 15). Водночас, після припинення ін'єкцій C_{60}FAS

його залишковий ефект у випадку *muscle gastrocnemius* становив 25-30% на першу добу і 17-23% на другу. Таку різницю між показниками у досліджуваних м'язах після застосування препарату вдалося зафіксувати, використовуючи лише біомеханічні маркери скорочення. Це свідчить про довготривалу кінетику його виведення з організму, яка сприяє тривалій (мінімум 48 год) компенсаторній активації C_{60} фулеренами ендогенної антиоксидантної системи у відповідь на стимуляцію м'яза, що необхідно враховувати при розробці нових фармпрепаратів на їх основі.

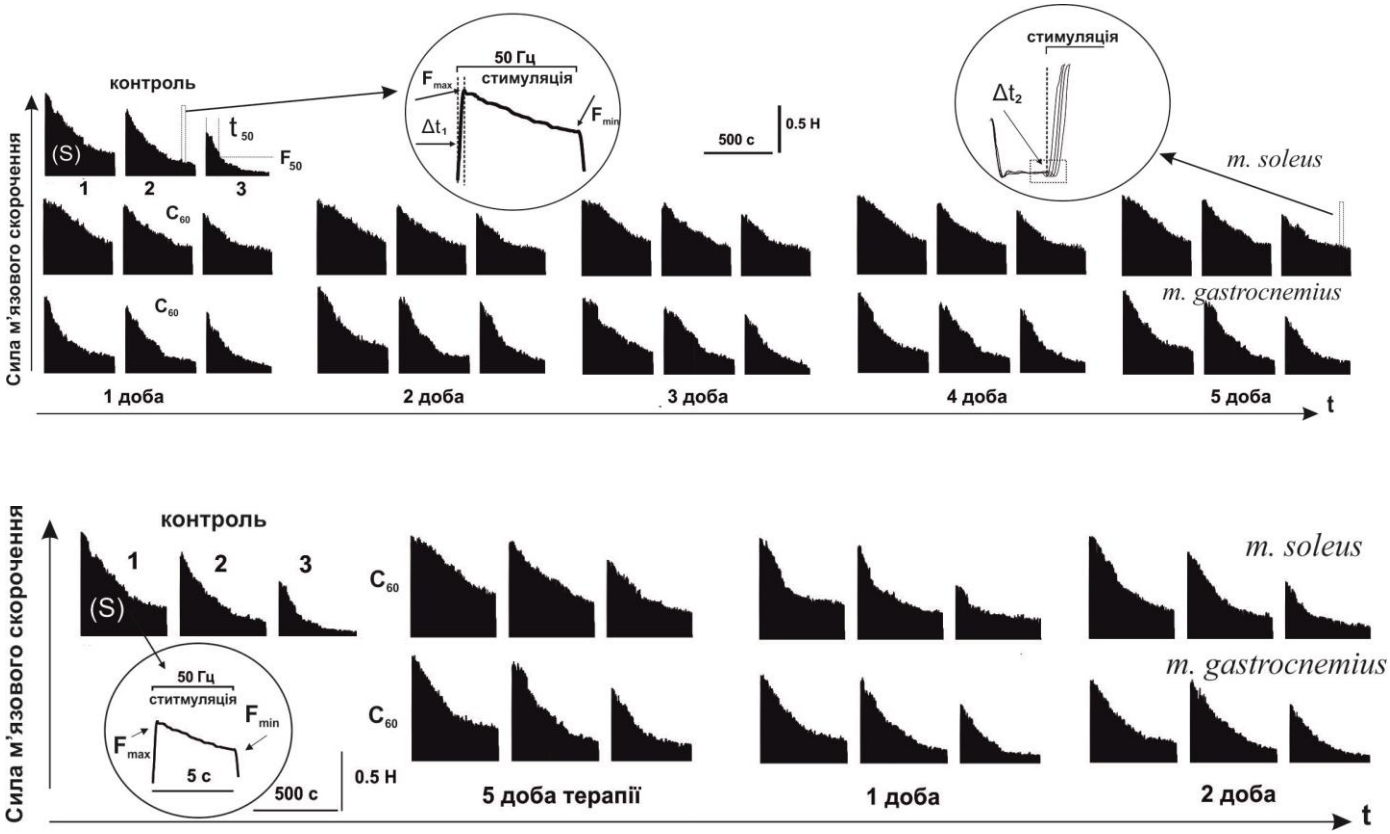

Рис. 15. Сила скорочення *muscle soleus* та *muscle gastrocnemius* щурівпісля введення C_{60}FAS (1 мг/кг) упродовж 1, 2, 3, 4 та 5 діб: S – імпульс м'язової сили; F_{max} та F_{min} – максимальна та мінімальна сила скорочення м'яза; Δt_1 – час досягнення максимальної сили; Δt_2 – час початку силової відповіді м'яза; t_{50} – час зменшення сили скорочення м'яза на 50% від максимального значення у пулі скорочень; 1 доба та 2 доба – механограми на 1 та 2 добу, відповідно, після закінчення застосування C_{60}FAS

Застосування C_{60}FAS змінило час зменшення сили скорочення *muscle gastrocnemius* щура на 50% від її максимального значення, яке становило 163%, 180% і 260% від контрольних значень на 1, 2 і 3 пулі стимуляції, відповідно. 5-ти добове застосування C_{60}FAS підвищило ці показники ще на 14-18% (рис. 15). Зміна часу

досягнення максимальної силової відповіді при застосуванні C_{60}FAS склала 75%, 92% і 119% від контролю, відповідно.

Важливо зауважити, що 5-ти добове застосування C_{60}FAS достовірно не змінювало цей показник, що може бути пов'язане з максимальним насиченням активного м'яза C_{60} фулереном вже в перший день його застосування для досягнення максимальної силової відповіді та встановлення оптимального дозового навантаження.

Ін'єкції C_{60}FAS призвели до зміни максимальної сили скорочення: ефект його застосування склав 15%, 54% і 63% на 1, 2 і 3 пулі стимуляцій, відповідно, порівняно з групою «патологія». Таким чином, ін’єкції C_{60} фулеренів підтримують стабілізацію механокінетики скорочувального процесу за тривалої активації досліджуваного м'яза. Показники мінімальної сили склали 58%, 109% і 121% після однодобового застосування та збільшилися на 13-16% після 5-ти добового введення C_{60}FAS порівняно з групою «патологія». Позитивний ефект C_{60}FAS на *muscle gastrocnemius* був на 20-25% більшим, чим на *muscle soleus*, що фіксували на усіх описаних маркерах. Максимальні ефекти застосування C_{60}FAS спостерігали на 3-му пулі стимуляції, де, у свою чергу, мали місце найбільші порушення у біомеханіці скорочення скелетного м'яза за розвитку його втоми. Слід відзначити, що описані особливості впливу C_{60}FAS на розвиток втомлювальних процесів можливо зафіксувати лише шляхом детального аналізу біомеханічних маркерів скорочення м’яза.

Біохімічні показники розвитку втомлювальних процесів (рис. 16) продемонстрували позитивний ефект ін’єкцій C_{60}FAS на рівні 25-30% у повільному м’язі та на 35-40% у швидкому порівняно з групою «патологія». Аналіз біохімічних показників крові на тлі індукованої м’язової втоми показав, що 5-ти добове застосування C_{60}FAS сприяє зниженню окисних процесів ще на 20-25% у швидких м’язах та на 15-19% у повільних порівняно з групою «патологія» завдяки підтриманню балансу між прооксидантами та системою антиоксидантного захисту. Порівняльний аналіз маркерів окисного стресу та показників стану системи антиоксидантного захисту засвідчив, що терапевтичний вплив C_{60}FAS на першу добу

(рис. 17) був вищим на 30-32% у швидких м’язах та на 35-40% у повільних порівняно з групою «патологія», причому ця різниця збільшується ще на 8-9% до 5-ї доби використання C_{60}FAS. Таким чином, тривале застосування C_{60}FAS сприяє зниженню окисних процесів у швидких і повільних м’язах завдяки підтриманню балансу між прооксидантами та системою антиоксидантного захисту, що запобігає негативному впливу АФК на клітинні та субклітинні структури за розвитку м’язової втоми у щурів.

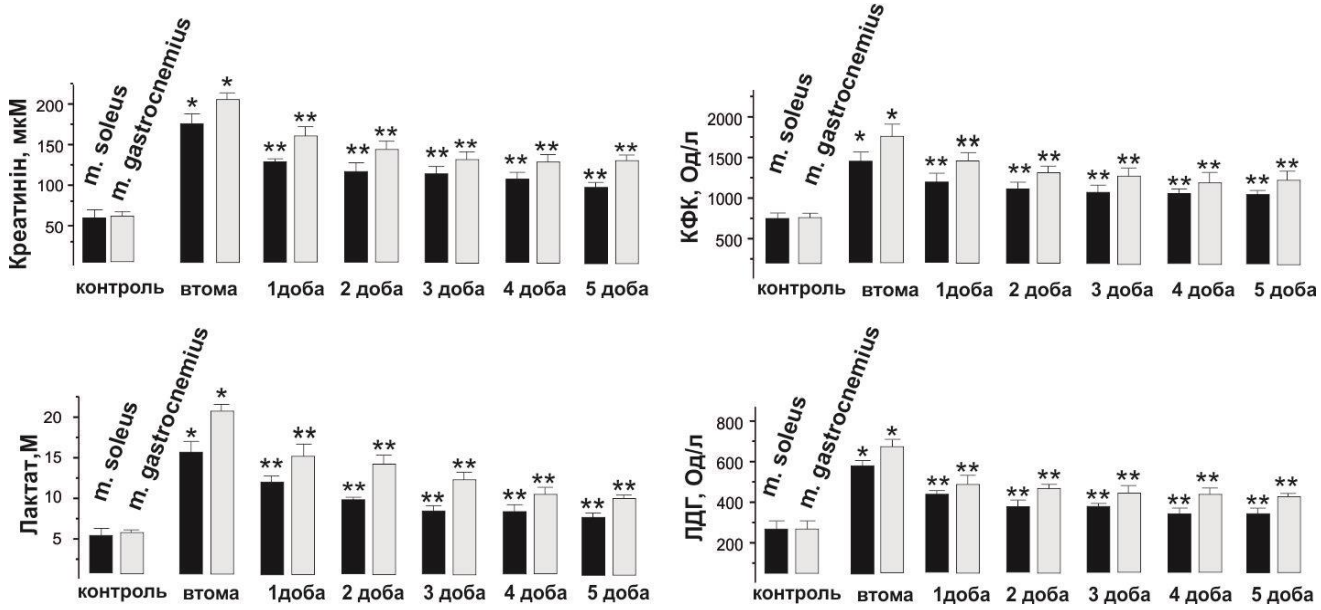

Рис. 16. Біохімічні показники розвитку втомлювальних процесів *muscle soleus* та *muscle gastrocnemius* щурів у крові після стимуляції та введення C_{60}FAS (1 мг/кг) упродовж 1, 2, 3, 4 та 5 діб. *p<0,05 щодо групи контроль; **p<0,05 щодо групи втома

Для оцінки ефективності дії C_{60}FAS за його введення до ініціації втоми скелетного м’яза було проведено порівняння рівня сили, що розвивається м'язами, за внутрішньом’язового та перорального введення препарату упродовж 5-ти діб. Відсутність статистично значущих відмінностей дозволило проводити подальший аналіз розвитку м’язової втоми лише з використанням перорального введення C_{60}FAS, оскільки воно є неінвазивним та практичнішим для застосування.

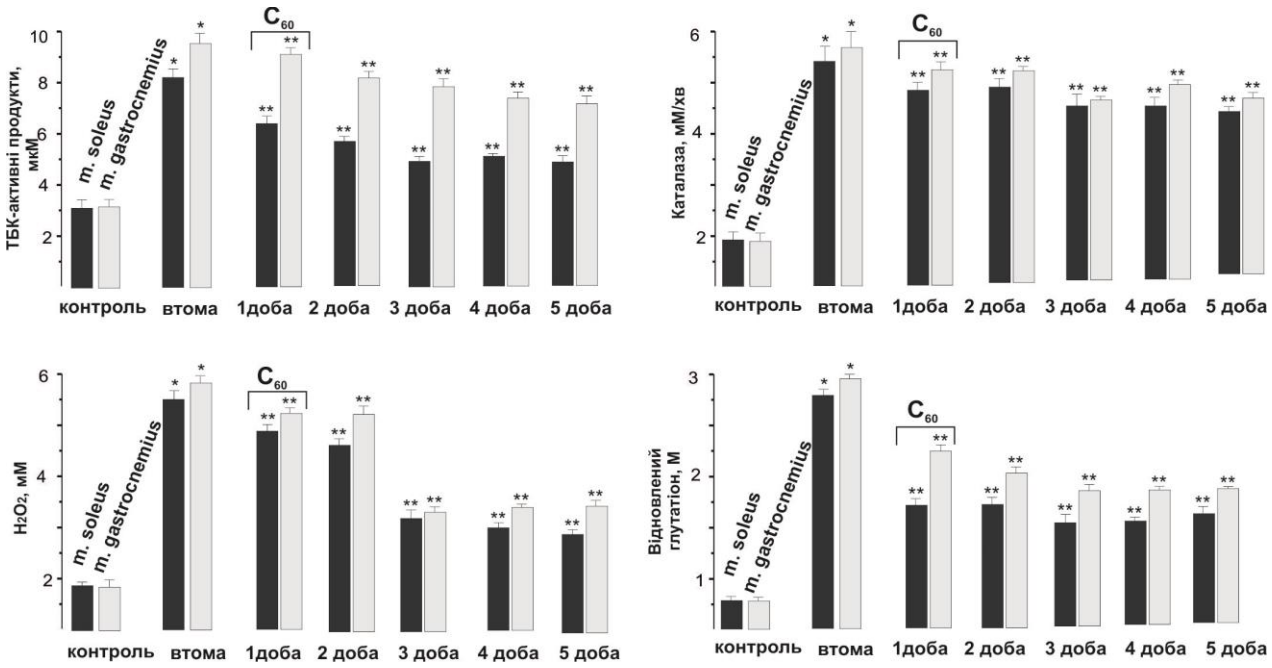

Рис. 17. Показники про-антиоксидантного балансу в крові щурів після стимуляції та введення C_{60}FAS(1 мг/кг) упродовж 1,2,3,4 та 5 діб. *p<0,05 щодо групи контроль; **p<0,05 щодо групи втома

Аналіз дії C_{60}FAS на силу скорочення скелетного м’яза за введення препарату до ініціації втоми засвідчив можливість м’язів підтримувати постійний рівень зусилля упродовж усіх п'яти застосованих серій стимуляції. Скелетні м'язи адекватно реагували на втомлювальні подразнення завдяки зменшенню рівня пошкоджень, що виникають внаслідок дії АФК. Накопичення вторинних продуктів у м'язових тканинах за застосування C_{60}FAS зменшилося на 25-35%порівняно з групою «патологія». Завдяки нанорозміру та мембранотропній активності водорозчинні C_{60} фулерени продемонстрували більш ефективний антиоксидантний вплив на гомеостаз м'язової тканини щурів, аніж відомі екзогенні антиоксиданти N-ацетилцистеїн і β-аланін, зокрема фіксували збільшення тривалості функціональної активності м’яза. Це відкриває реальну можливість для перорального застосування C_{60}FAS як потенційного агента корекції втомлювальних процесів скелетних м’язів.

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4. **D. Nozdrenko**, S. Prylutska, K. Bogutska, V. Cherepanov, A. Senenko, O. Vygovska, S. Khrapatyi, U. Ritter, Yu. Prylutskyy, J. Piosik. Analysis of Biomechanical and Biochemical Markers of Rat *Muscle Soleus* Fatigue Processes Development during Long-Term Use of C_{60} Fullerene and N-Acetylcysteine. Nanomaterials, 2022; 12(9): 1552. **(Q1)**

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РОЗДІЛ 4. *Механокінетичні параметри скорочення скелетних м'язів та біохімічні показники крові щурів після м'язової атрофії, спричиненої ахіллотенотомією*

Функціональне розвантаження скелетних м'язів, викликане знерухомленням, здатне викликати їхню атрофію. При цьому найбільш глибокі атрофічні зміни спостерігаються у камбалоподібному – ключовому постуральному м'язі. Функціональне розвантаження призводить до пригнічення синтезу білка та активації протеолізу, що виявляється у зменшенні діаметра м'язових волокон (атрофія) та втраті їх сили скорочення. Атрофія м'язових волокон виходить на плато приблизно через 14 діб знерухомлення або гравітаційної бездіяльності. Прогресуючий розвиток патологічних процесів у м'язових волокнах триває після їх знерухомлення упродовж 30-40 діб. Для імітації невикористання задніх кінцівок щурів традиційно використовують модель розриву ахіллового сухожилля (ахіллотенотомія). Значна атрофія литкового м'яза фіксується, починаючи з 10-ї доби дослідження. За час терапевтичних процедур та реабілітаційного періоду відновлення завжди має місце деградація м'язового апарату. Таким чином, визначення ступеня тяжкості патології, на якому базується розробка протоколу реабілітації, є невід'ємним аспектом відновлення рівня м'язової активності. Останнім часом застосування антиоксидантної терапії на ранніх стадіях розвитку атрофії м'язів демонструє перспективність цього напряму досліджень. Застосування низки антиоксидантів (куркуміну, глабридіну та флавоноїдних сполук) веде до зниження окислювального стресу та активності протеолітичних шляхів і, як наслідок, зменшує деградацію м'язового білка за розвитку атрофії м'яза [Mareen, et al., 2014]. Ці дані стали основою для проведення тестування водорозчинних C_{60} фулеренів як потенційних агентів, що зменшують патологічні ефекти у м'язовій системі щурів за розвитку м'язової атрофії.

На 15 добу після ініціації ахіллотенотомії максимальна сила скорочення *muscle soleus* щурів знизилася до 58% відносно контрольної групи. Таким чином, має місце різке зниження силової активності м'яза вже на перших скороченнях з прогресивним зниженням досліджуваних біомеханічних показників (рис. 18). На 30 і 45 добу після

ахіллотенотомії показники максимальної силової відповіді зменшилися до 79% та 88%, відповідно. За щоденного перорального вживання C_{60}FAS у дозі 1 мг/кг ці показники становили 65% на 15, 84% на 30 і 95% на 45 добу порівняно з групою «патологія». Зменшення імпульсу м'язової сили на 15 добу після ініціації патології склало 41%. На 30 і 45 добу ці показники склали 70% і 84%, відповідно. При застосуванні C_{60}FAS позитивний ефект становив у середньому 35-40%порівняно з групою «патологія». Час досягнення максимальної силової на 15 добу після активації збільшився на 97%. На 30 і 45 добу ці показники склали 56% і 42%, відповідно. При застосуванні C_{60}FAS зафіксована корекція цих показників: позитивний ефект становив у середньому 50-60% на 15 добу та 20-25% на 45 добу порівняно з групою «патологія» (рис. 18).

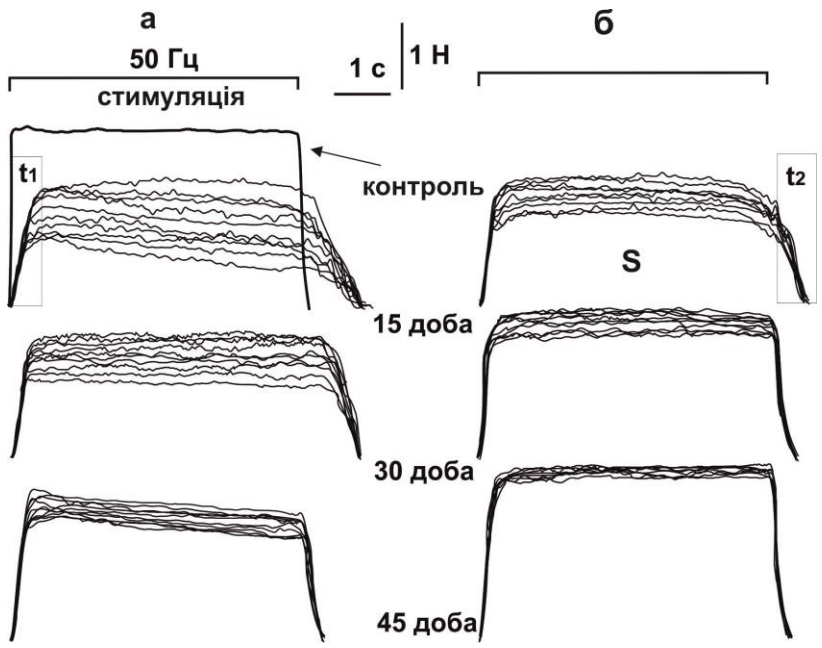

Рис. 18. Сила скорочення *muscle soleus* після розвитку атрофії, викликана 10-ти послідовними 6-ти с безрелаксаційними пулами стимуляції: без введення C_{60}FAS (а) та за введення C_{60}FAS у дозі 1 мг/кг (б); нативний м'яз – контроль; t_1 – час розвитку максимальної силової відповіді; t_2 – час відновлення силових параметрів до початкових значень; S – імпульс м'язової сили

Цей факт можна пояснити тим, що патологічні фактори, що впливають на час досягнення максимальної силової відповіді, активуються в перші дні після знерухомлення і знижують свій патологічний вплив при збільшенні часу після описаної травми. Прогресуюче зниження силової відповіді триває щонайменше 15 діб, після чого починається процес відновлення м’яза.

Час відновлення силових параметрів до початкових значень напряму залежить від жорсткості м'яза та еластичних властивостей сухожильних компонентів. На 15 добу після активації атрофії зафіксовано його збільшення на 38%. На 30 і 45 добу ці показники склали 25% і 19%, відповідно. При застосуванні C_{60}FAS зафіксовано зменшення часу відновлення силових параметрів до початкових значень на 25-33% упродовж експерименту.

Таким чином, отримані дані свідчать про позитивну динаміку застосування водорозчинних C_{60}фулеренів у щоденній дозі 1 мг/кг, що веде до зниження тяжкості м'язового пошкодження упродовж експерименту.

Збільшення кількості внутрішньом'язової сполучної тканини внаслідок знерухомлення має ключове значення для виникнення підвищеної м’язової втоми. Тому наступним етапом наших досліджень був аналіз виникнення втомлювальних процесів у *muscle soleus* після розвитку атрофії. Реєстрація сили скорочення м’яза при застосуванні втомлювальної стимуляції засвідчила зменшення величини імпульсу м’язової сили на 28%, 59% і 64% щодо контрольної групи на 15, 30 і 45 добу експерименту, відповідно (рис. 19). Позитивний ефект склав понад 50% порівняно з групою «патологія»упродовж експерименту, що, на нашу думку, пов'язано з антиоксидантними властивостями C_{60} фулеренів корегувати втомлювальні процеси в активному м'язі. Час зменшення силової відповіді на 50% від початкових значень при застосуванні C_{60}FAS показав його 35-40% позитивний ефект на етапах утримання максимальних силових відповідей упродовж розвитку втомлювальних процесів. При застосуванні стимуляційних пулів наростаючої частоти *muscle soleus* після ініціації атрофії упродовж усього експерименту так і не вийшов на стадію гладкого тетанічного скорочення. Застосування C_{60}FAS змінило біомеханічні параметри переходу *muscle soleus* із зубчастого в гладкий тетанус, який виникав з 16% різницею

між контрольними значеннями (рис. 20). Застосування C_{60}FAS виявили позитивну динаміку на максимальну та мінімальну сили поодинокого скорочення на рівні 15-19% порівняно з групою «патологія»упродовж експерименту.

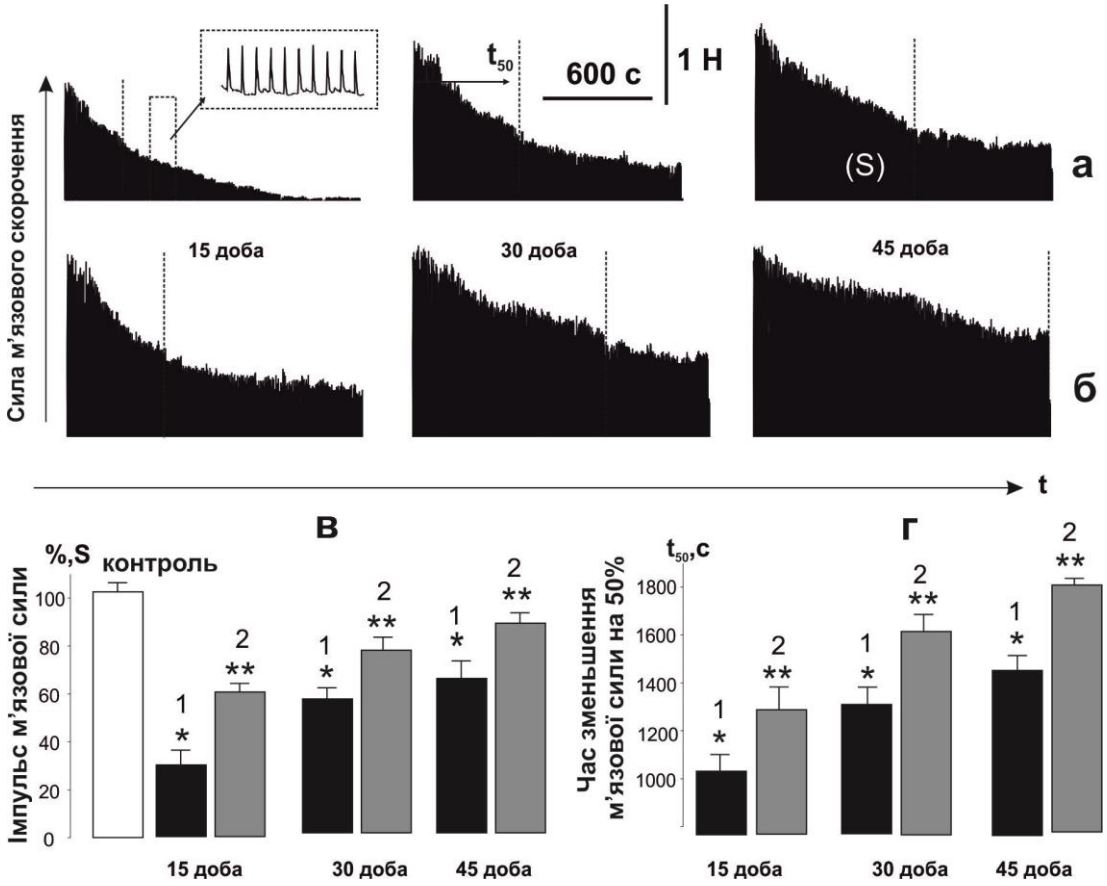

Рис. 19. Біомеханічні параметри *muscle soleus* після атрофії при застосуванні 1 Гц стимуляції тривалістю 1800 с: без введення C_{60}FAS (а); при введенні C_{60}FAS у дозі 1 мг/кг (б); імпульс м'язової сили (S), представлений у відсотках від значень контрольної групи (в); час зменшення силової відповіді на 50% початкових значень (t_{50}) (г). Нативний м'яз – контроль; 1, 2 – відповідні значення параметрів без введення C_{60}FAS та при його застосуванні, відповідно. *$p<0,05$ порівняно з контрольною групою; **$p<0,05$ порівняно з групою без застосування C_{60}FAS

Зміни маси камбалоподібного м'яза, величин максимальної сили поодинокого тетанічного скорочення ізольованого м'яза та його площі поперечного перерізу після атрофії відображають рівень деструкції м'язової тканини. Маса камбалоподібного

м'яза, нормована на масу тіла тварини, у групах, що вживали C_{60}FAS, була в середньому на 35-37% більшою, ніж у патологічній групі упродовж експерименту.

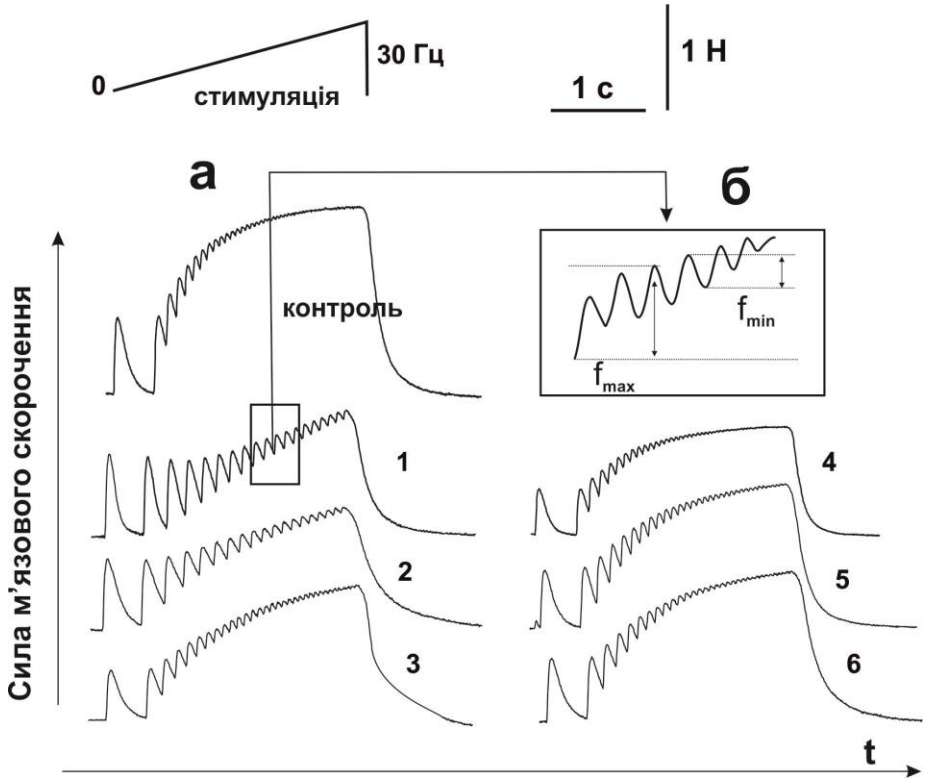

Рис. 20. Механограми переходу *muscle soleus* після атрофії із зубчастого в гладкий тетанус при застосуванні наростаючої стимуляції максимальної частоти 30 Гц тривалістю 6 с: без введення C_{60}FAS (а); при введенні C_{60}FAS у щоденній дозі 1 мг/кг (б). Нативний м'яз – контроль; f_{max} – максимальна сила поодинокого скорочення; f_{min} – мінімальне значення силової відповіді в одному зубці зубчастого тетанусу; 1, 2, 3 – значення відповідних параметрів на 15, 30 і 45 добу після атрофії, відповідно, без введення C_{60}FAS; 4, 5, 6 - значення відповідних параметрів на 15, 30 і 45 добу після атрофії, відповідно, при застосуванні C_{60}FAS

Застосування C_{60}FAS збільшило максимальну силу поодинокого тетанічного скорочення на 30%, 32% і 36% на 15, 30 і 45 добу, відповідно, порівняно з групою «патологія» (Табл. 1). Найбільш суттєві результати показали зміни середньої максимальної тетанічної сили (P_0), нормованої на площу поперечного перерізу м'яза

(P_0/S). При використанні C_{60}FAS ці показники більш ніж на 40% були вищими, ніж у попередній групі упродовж експерименту.

Табл. 1.

Маси тіла тварин і камбалоподібного м'яза, значення середньої максимальної тетанічної сили (P_0), маси камбалоподібного м'яза, нормованої на масу тіла тварини, та середньої максимальної тетанічної сили, нормованої на площу поперечного перерізу м'яза (P_0/S), на 15, 30 і 45 добу після атрофії.

Групи тварин	**Маса тварини, г**	**Маса *muscle soleus*, мг**	**Маса *muscle soleus*/Маса тварини**	**P_0, мН**	**P_0/S,Н/см^2**
контроль	205±8	102,4±1,5	0,49±0,01	882,4±14,3	23,4±1,2
15 доба	231±6*	63,4±1,8*	0,27±0,03*	432,5±16,1*	14,4±2,5*
30 доба	243±4*	73,4±1,2*	0,30±0,02*	676,5±11,6*	17,6±7,3*
45 доба	250±6*	86,4±1,5*	0,34±0,02*	693,3±14,1*	18,6±4,4*
15 доба+ C_{60}	244±5**	79,4±1,2**	0,32±0,02**	602,5±12,2**	18,1±1,2**
30 доба+ C_{60}	254±2**	89,4±1,3**	0,35±0,02**	711,5±22,5**	19,2±1,1**
45 доба+ C_{60}	269±7**	105,4±1,9**	0,39±0,05**	782,5±16,3**	20,3±1,2**

*p<0,05 порівняно з контрольною групою; **p<0,05 порівняно з групою без введення C_{60}FAS

За отриманими даними можна зробити висновок, що введення C_{60}FAS у щоденній дозі 1 мг/кг знижує рівень деструкції м'язової тканини на 30-35% порівняно з групою «патологія»упродовж експерименту. У всіх проведених тестах відбувається позитивна зміна біохімічних показників розвитку патологічних процесів у *muscle soleus* приблизно на 27-30% (рис. 21) та на 21-23% за показниками про- та антиоксидантного балансу (рис. 22) при введенні C_{60}FAS порівняно з групою «патологія». Це свідчить про наявність компенсаторної активації водорозчинними C_{60} фулеренами ендогенної антиоксидантної системи в процесі деструкційних змін *muscle soleus*, спричиненої атрофією.

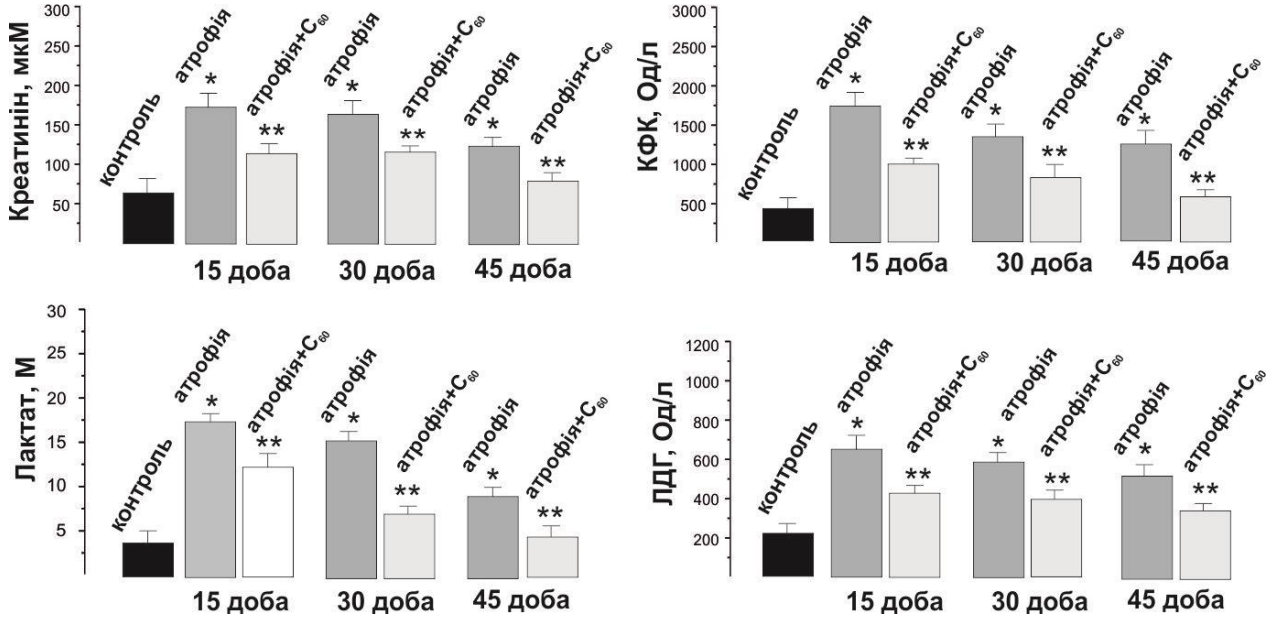

Рис. 21. Біохімічні показники розвитку патологічних процесів у крові щурів після застосування 1 Гц стимуляції *muscle soleus* тривалістю 1800 с на 15, 30 і 45 добу після атрофії. *p<0,05 порівняно з групою контроль; **p<0,05 порівняно з групою без введення C_{60}FAS

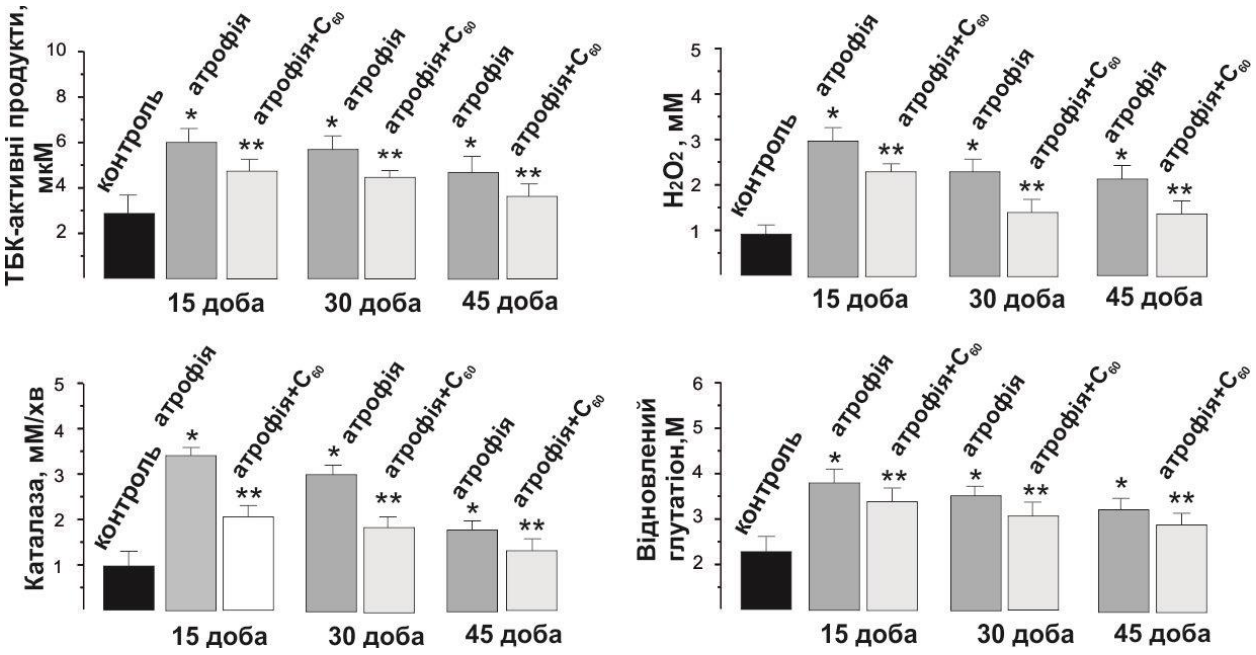

Рис. 22. Показники про- та антиоксидантного балансу в крові щурів після застосування 1 Гц стимуляції *muscle soleus* тривалістю 1800 с на 15, 30 і 45 добу після атрофії. *p<0,05 порівняно з групою контроль; **p<0,05 порівняно з групою без введення C_{60}FAS

Таким чином, можна стверджувати, що позитивні зміни досліджуваних біомеханічних і біохімічних маркерів підтверджують можливість застосування C_{60}FAS як перспективного агента, здатного зменшувати і корегувати патологічні стани м'язової системи, що виникають за атрофії скелетних м'язів. Використання вище описаних біомеханічних маркерів повністю корелює з біохімічними маркерами розвитку цієї патології.

Основні публікації за цим розділом:

1. **D. Nozdrenko,** S. Prylutska, K. Bogutska, N. Nurishchenko, O. Abramchuk, O. Motuziuk, Yu. Prylutskyy, P. Scharff, U. Ritter. Effect of C_{60} Fullerene on Recovery of *Muscle Soleus* in Rats after Atrophy Induced by Achillotenotomy. Life, 2022, 12(3): 332. **(Q2)**

УЗАГАЛЬНЕННЯ

Представлені результати дослідження біомеханічних параметрів скорочення скелетних м’язів та біохімічних показників крові щурів за різних експериментально-індукованих м’язових патологій (зокрема ішемії, механічної травми, втоми та атрофії) різного ступеня тяжкості (1, 2 і 3) та дії C_{60}FAS у різних часових (1-45 діб залежно від патології) і дозових (0,5, 1 і 2 мг/кг ваги тварини) діапазонах залежно від способу (внутрішньом'язове, пероральне) та схеми його введення (за 1 год до і після ініціації м’язової патології). Використання для аналізу описаних процесів біомеханічних маркерів амплітудно-швидкісних змін силової відповіді продемонструвало позитивний ефект C_{60}FAS на рівні понад 50% на маркери точного позиціонування, тоді як біохімічні зміни крові не перевищували 10% рівня порівняно з групою «патологія». Важливо, що використання для аналізу досліджуваних патологічних процесів саме біомеханічних маркерів амплітудно-швидкісних змін силової відповіді м’яза виявило кількісні відмінності між ними як при зростанні ступеня тяжкості патології, так і при застосуванні C_{60}FAS у різних дозових і часових діапазонах, що дає можливість відтворити у певному хронологічному порядку алгоритм їх «спрацювання». Запропоновані універсальні біомеханічні маркери м'язового

скорочення мають різну чутливість до ступеня тяжкості патології, що розвивається, і вибудовуються у такому порядку (рис. 23: м’язова патологія): 1 - мінімальна сила поодинокого скорочення м'яза (F_{min}); 2 - час досягнення максимальної сили скорочення м'яза (t_{max}); 3 - максимальна сила поодинокого скорочення м'яза (F_{max}); 4 - імпульс м’язової сили (S); 5 - час зменшення сили скорочення м'яза на 50% (t_{50}); 6 - час між початком стимуляції і початком скорочення м'яза (t_{start}); 7 - час після припинення стимуляції до виходу м'язової сили на початковий рівень (t_0).

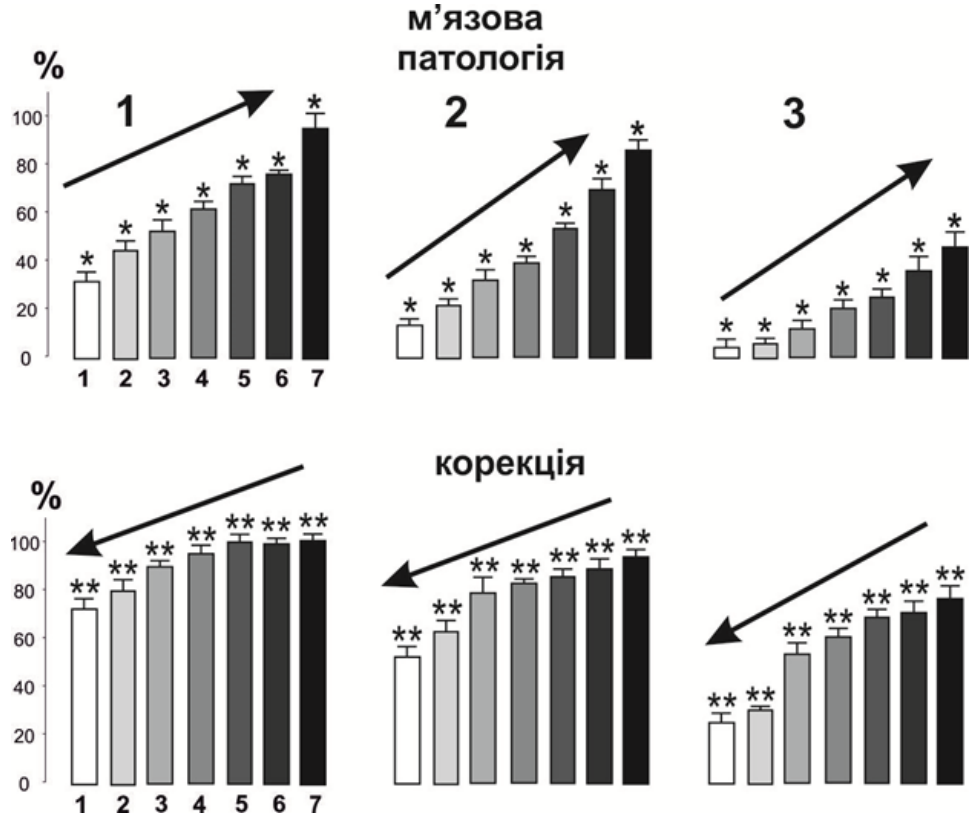

Рис. 23. Хронологічний порядок «спрацювання» біомеханічних маркерів амплітудно-швидкісних змін силової відповіді скелетного м’яза (1, 2, 3, 4, 5, 6, 7) за різного ступеня тяжкості патології (м’язова патологія: 1, 2 і 3) та дії C_{60}FAS (корекція)

З іншого боку, позитивні зміни біохімічних показників крові за досліджуваних м’язових патологій свідчать про активацію антиоксидантної реакції організму щурів у відповідь на введення C_{60}FAS. Це може відбуватися двома шляхами: зменшенням кількості вільних радикалів завдяки їх адсорбції молекулами C_{60} (чи їх наноагрегатами), або безпосереднім впливом на величини біохімічних показників за

умов розвитку стресових станів. C_{60} фулерен послаблює утворення АФК і, таким чином, зменшує ПОЛ, а також підвищує антиоксидантну здатність тканин щурів. Зокрема, він підвищував вміст відновленого глутатіону (GSH) та активність/експресію білка GSH-пов'язаних ферментів. Кореляція цих змін із вмістом білка Nrf2 свідчить про те, що під впливом стресу разом з іншими механізмами Nrf2/ARE-антиоксидантний шлях може брати участь у регуляції гомеостазу глутатіону.

Нарешті, зменшення ступеня тяжкості патологічного стану за дії C_{60}FAS відображає послідовність «спрацювання» описаних біомеханічних маркерів амплітудно-швидкісних змін силової відповіді скелетного м'яза у зворотному порядку (рис. 23: корекція), що дає можливість використовувати запропонований алгоритм для визначення рівня м'язового пошкодження при контролі ефективності терапевтичних і реабілітаційних процедур.

ВИСНОВКИ

У дисертаційній роботі представлені результати комплексного дослідження механокінетичних параметрів скорочення м'язів щурів та біохімічних показників їх крові за різних експериментально-індукованих м'язових патологій різного ступеня тяжкості та дії водорозчинних вуглецевих наночастинок - C_{60} фулеренів у різних часових і дозових діапазонах залежно від способу та схеми їх застосування. Зокрема показано, що водорозчинні C_{60} фулерени здатні ефективно зменшувати негативну дію АФК, індукованих тією чи іншою м'язовою патологією, сприяючи антиоксидантній системі організму впоратися з прогресуючим розвитком патологічних процесів.

Встановлені універсальні механокінетичні маркери м'язової відповіді, на основі яких можливо визначити ступінь тяжкості перебігу цих патологій.

1. Показано, що введення водного розчину C_{60} фулеренів (C_{60}FAS) до ішемізації скелетного м'яза за оптимальної дози 1 мг/кг збільшує силову відповідь м'яза на 65%, 40% та 35% у випадку 1, 2 та 3 ступеня тяжкості ішемії, відповідно, порівняно з групою «патологія». Водночас, рівень біохімічних маркерів у крові щурів знижується

в середньому на 30%, 25% та 20% у випадку 1, 2 та 3 ступеня тяжкості ішемії м’яза, відповідно, порівняно з групою «патологія».

Введення C_{60}FAS після ініціації цієї патології продемонструвало достовірно менший ефект, який за максимальних значень досліджуваних маркерів м’язових пошкоджень відрізнявся на 20-25% порівняно з тими параметрами, що спостерігали у випадку введення C_{60}FAS до ішемізації скелетного м’яза.

2. Показано, що введення C_{60}FAS до травмування скелетного м’яза за оптимальної дози 1 мг/кг збільшує силову відповідь м'яза на 21%, 19% та 12% у випадку 1, 2 та 3 ступеня тяжкості м'язової травми, відповідно, порівняно з групою «патологія». Водночас, зменшення рівня біохімічних маркерів у крові щурів на 10-12% за цієї патології підтверджує позитивний ефект C_{60}FAS.

Введення C_{60}FAS після ініціації цієї патології продемонструвало достовірно менший ефект, який не перевищував 40% від значень досліджуваних маркерів м’язових пошкоджень у випадку введення C_{60}FAS до травмування скелетного м’яза.

3. Показано, що введення C_{60}FAS після ініціації м’язової втоми за оптимальної дози 1 мг/кг веде, з одного боку, до зменшення часу відновлення сили скорочення м’яза, а з іншого, збільшує час його активного функціонування. Ефект C_{60}FAS для *muscle gastrocnemius* на 30% більший, ніж для *muscle soleus*. Введення C_{60}FAS упродовж 5-ти діб знижує ступінь тяжкості втомлю вальних процесів на 40% у повільному м'язі та на 60% у швидкому порівняно з групою «патологія». Залишкова дія C_{60}FAS складала 25% на 1 добу і 15% на другу порівняно з групою «патологія». Зменшення рівня біохімічних маркерів у крові щурів на 25-30% у повільному м’язі та 35-40% у швидкому порівняно з групою «патологія» підтверджує позитивний ефект C_{60}FAS.

Введення C_{60}FAS до ініціації цієї патології продемонструвало його значний захисний ефект, який вже на 1-у добу експерименту на 14-19% перевищував значення досліджуваних параметрів, які спостерігали у випадку введення C_{60}FASпісля ініціації м’язової втоми.

4. Показано, що введення C_{60}FAS після ініціації атрофії скелетного м’яза за оптимальної дози 1 мг/кг збільшує його силову відповідь на 25-35% порівняно з

групою «патологія». Зменшення рівня біохімічних маркерів у крові щурів на 27-30% за цієї патології підтверджує позитивний ефект C_{60}FAS.

Нарешті, доведено, що біомеханічні маркери амплітудно-швидкісних змін силової відповіді м'яза мають різну чутливість до ступенів тяжкості досліджуваних патологій і вибудовуються у такому хронологічному порядку: F_{min} - t_{max} - F_{max} - S - t_{50} - t_{start} - t_0. Виявлена позитивна кореляція між біохімічними маркерами у плазмі крові щурів та вищевказаними біомеханічними маркерами за розвитку м'язових патологій уможливлює їх цілеспрямоване використання для визначення ступеня тяжкості та ефективності застосованих терапевтичних і реабілітаційних процедур.

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Праці, в яких опубліковані основні наукові результати дисертації

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2. Yu.I. Prylutskyy, 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. C_{60} fullerene as promising therapeutic agent for correcting and preventing skeletal muscle fatigue. Journal of Nanobiotechnology, 2017, 15(1): 8. **(Q1)**

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4. D. Nozdrenko, T. Matvienko, O. Vygovska, K. Bogutska, O. Motuziuk, N. Nurishchenko, Yu. Prylutskyy, P. Scharff, U. Ritter. Protective effect of water-soluble C_{60} fullerene nanoparticles on the ischemia-reperfusion injury of the *muscle soleus* in rats. International Journal of Molecular Sciences, 2021, 22(13): 6812. **(Q2)**

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8. **D. Nozdrenko,** S. Prylutska, K. Bogutska, N. Nurishchenko, O. Abramchuk O. Motuziuk, Yu. PrylutskyyP. Scharff, U. Ritter. Effect of C_{60} fullerene on recovery of *muscle soleus* in rats after atrophy induced by achillotenotomy. Life, 2022,12(3): 332. **(Q2)**

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Праці, які додатково відображають наукові результати дисертації

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C_{60} Fullerene as Promising Therapeutic Agent for the Prevention and Correction of Skeletal Muscle Functioning at Ischemic Injury

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Abstract

The therapeutic effect of pristine C_{60} fullerene aqueous colloid solution (C_{60}FAS) on the functioning of the rat *soleus muscle* at ischemic injury depending on the time of the general pathogenesis of muscular system and method of administration C_{60}FAS in vivo was investigated. It was found that intravenous administration of C_{60}FAS is the optimal for correction of speed macroparameters of contraction for ischemic muscle damage. At the same time, intramuscular administration of C_{60}FAS shows pronounced protective effect in movements associated with the generation of maximum force responses or prolonged contractions, which increase the muscle fatigue level. Analysis of content concentration of creatine phosphokinase and lactate dehydrogenase enzymes in the blood of experimental animals indicates directly that C_{60}FAS may be a promising therapeutic agent for the prevention and correction of ischemic-damaged skeletal muscle function.

Keywords: C_{60} fullerene, Skeletal muscle, Ischemia, Muscle contraction dynamics, Biochemical analysis

Background

Among muscle pathologies that develop in the skeletal muscles, the ischemic injuries are more than 35% of all injuries of musculoskeletal system [1]. Ischemic-reperfusion injury of skeletal muscle is a major cause of postoperative pathologic complications [2], particularly, the reason of amputations and mortality is acute arterial occlusion [3]. Due to the delivery reduction of oxygen in blood flow through the blood vessels, the nutrients and regulatory substances cannot reach; thus, the muscle decreases. This can lead to a progressive disorder in its metabolic, morphological, and physiological processes.

The main aim in the treatment of the ischemic muscles is the fast recovery of blood flow (reperfusion) in the damaged areas. However, this therapy often leads to new pathophysiological process; reperfusion injury, which also can cause significant damage in the muscle tissue.

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At ischemic injury of the skeletal muscle, there is a high correlation between the duration of ischemia and survival of muscle fiber [4]. Despite the fact that different types of fibers in the skeletal muscle differ from the metabolic and functional properties, it has no significant impact on their tolerance to ischemia-reperfusion injuries [5].

At the biochemical level, the ischemic damage of the muscle tissue is a sequence of biochemical reactions, which are initiated by hypoxia after a few minutes of ischemia and occur independently of etiological features due to insufficient blood supply to the muscle [1]. The death of the majority of the muscle cells is a result of chemical substance activation, which are produced during and after ischemia and can be formed within a few days even after the restoration of normal blood flow to the muscles.

It is known that after 2 h of skeletal muscle ischemia and further reperfusion, the concentration of ATP significantly reduced simultaneously with a significant increase in the number of lactate from 25 to 114 mmol/kg of dry weight. And after 3 h of ischemia, the intramuscular supply of ATP is about 5% from baseline, and glycogen pool is depleted by

88% [6]. From a functional point of view, these data indicate that a large number of high-energy phosphate compounds in ischemic-damaged muscle cells are spent to maintain homeostasis (especially during the first hour of ischemia) and, consequently, metabolic causes a significant increase in the fatigue ischemic muscle [7].

It is known that free radicals are a major pathogenic factor in the development of ischemic damage in the muscle tissue [8]. Preliminary biological studies of water-soluble pristine C_{60} fullerenes [9–13] have shown that at low (physiological) concentrations, they do not exhibit acute toxic effects on the normal cells [14–16], they are not allergenic and immunogenic and they able to regulate free-radical processes in the cells and tissues, in particular, neutralize excess free radicals [17, 18]. Consequently, the use of biocompatible and bioavailable C_{60} fullerenes as powerful antioxidants [19] opening up new potential opportunities for the prevention and correction of ischemic-reperfusion pathological processes in the muscle tissue.

The purpose of this study was to assess the impact of water-soluble pristine C_{60} fullerenes on mechanical and kinetic peculiarities of rat skeletal muscle function at ischemic injury, namely: (1) to conduct a quantitative analysis of the activity of ischemic-damaged muscle structures and establish a link between the change in mechano-kinetics of physiological contractions and the level of C_{60} fullerenes action that is necessary for this change and (2) to evaluate the therapeutic effect of C_{60} fullerenes on the time of development of general pathogenesis of muscular system depending on the method of administration (intravenous and intramuscular) in vivo.

Methods

A highly stable reproducible pristine C_{60} fullerene aqueous colloid solution (C_{60}FAS) in concentration 0.15 mg/ml was prepared and characterized according to the protocol [20, 21].

The study was conducted on white male rats of the "Wistar" line weighing 170 ± 5 g. The animals were kept under standard conditions in the vivarium of the ESC "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv. Animals had free access to food and water. All experiments were conducted in accordance with the international principles of the European Convention for protection of vertebrate animals under a control of the Bio-Ethics Committee of the abovementioned institution.

All experimental animals were divided into four groups: intact group (animals with saline injection; $n = 10$), control group (animals after ischemia without C_{60}FAS injection; $n = 10$), and two experimental groups (animals after ischemia with C_{60}FAS injection intravenously ($n = 10$) and intramuscularly (n = 10) immediately after reperfusion). For therapeutic purposes we used C_{60}FAS in a concentration of 1 mg/kg because this dose was the most effective at muscular therapy [22, 23].

Anesthesia of animals was performed by intraperitoneal administration of nembutal (40 mg/kg). For muscle ischemia, the branch of the femoral artery of the animal, which provides blood supply of the experimental muscle, was dragged by ligatures. Standard preparation of the experiment also included the cannulation (a. carotis communis sinistra) for the therapeutic administration of C_{60}FAS and pressure measurement, tracheotomy, and laminectomy at lumbar spinal cord level. *Soleus muscle* of rat was released from the surrounding tissues and its tendon was cut across in distal part. The ventral roots were cut in places of their exit from the spinal cord for the modulated stimulation of efferents in L7-S1 segments.

The change in muscle contraction force was measured using the original strain gages [22, 23]. To generate stimulus signals, the programmable generator of signals of special form was used.

The study of dynamic properties of muscle contraction was performed under conditions of muscle activation using the modulated stimulation of efferents. Five filaments of ventral roots were cut and fixed on stimulating electrodes, and a special device was used for cyclic sequence distribution of electrical signals via the filaments [22, 23]. The distributed stimulation was allowed to get the monotonous and uniform muscle contraction at low stimulation frequency of individual filaments (50 Hz). Stimulation of efferents in L7-S1 segments was performed by electric impulses of 2 ms, formed by using a pulse generator controlled by ACC through the platinum electrodes. The parameters of stimulated signal were programmed and transmitted from the ACC-CAC complex to generator. A control of external load on the muscle was carried out with the help of original mechanical stimulator [23, 24]. The electromagnetic linear motor was used for perturbation load.

The muscle contraction force was measured at 1, 2, 3, 4, and 5 experimental hours and at 1, 2, 3, 4, and 5 experimental days after ischemia. All received force curves reflect the change in the percentage of control values of the intact muscles, which were taken as 100%.

Level of content of creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) enzymes in the blood of experimental animals, as the markers of ischemic injury of the skeletal muscle, was determined by using a clinical equipment.

The experimental data were stored and analyzed by statistical processing of the results using standard software packages Excel and Origin 8.0. All results were expressed as mean ± SEM. The significance of differences of baseline values between control and experimental groups was evaluated by t test. A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Biomechanical Study

Kinetics of muscle fatigue and change of the force response in each flow of muscle contraction induced by stimulation successive pools are important characteristics of the pathogenesis of muscle ischemic injury study. Under normal conditions, the fatigue changes during the contraction of the *soleus muscle* detected only after 5–6 h stimulation [25].

The main processes, which initiate a cascade of ischemic pathologies in the damaged muscle, occur in the first hours after reperfusion [26]. Based on this, the first step in research is to examine the change in the dynamics of the contractile process in the first 5 h after reperfusion of the ischemic *soleus muscle*. Comparing intravenous and intramuscular administration of C_{60}FAS, we tried to determine the optimal method of its administration to achieve maximum therapeutic effect.

Figure 1 shows change in the *soleus muscle* force response during the first 5 h after its reperfusion under activating stimulus pools with duration from 2 to 5 s.

In the control (without affecting C_{60}FAS), reduction of maximal force responses not only with an increasing time after ischemia but also with increasing duration of irritating stimulus signal is observed (Fig. 2). In case of therapeutic administration of C_{60}FAS, the reduction of force response with increasing of time irritating signal is negligible and depends mainly on time after reperfusion (Fig. 2). It is important to note that in this case the method of administration of C_{60}FAS had no significant value.

The registration of such important biomechanical parameter as integrated power (it's calculated by total area, which describes the force curve) found the similar results (Fig. 2): the integrated power reduction as with an increasing time after reperfusion and with increasing duration stimulation signal is largely compensated by the influence of C_{60}FAS regardless of its method of administration. It is also important to note that the protective effect of C_{60}FAS manifests on the first hours of ischemic muscle injury, during which the initiation of the main stages of ischemic destruction of the muscle tissue takes place.

Based on the fact that muscle contraction is a dynamic vibrational process of mutual reactions, one can assume that in the conditions of pathological changes in muscle fibers caused by ischemia, there should be optimum stimulation parameter ratio, which can involve the maximum number of sarcomere structures for the optimal muscle contraction. Although the heterogeneous composition of skeletal muscle contractile apparatus is difficult to assess the damage of each individual component, the overall picture of the pathological process can be traced by measuring the level of changes of maximum force contraction for several days (Figs. 3 and 4). In control, the muscle activity had a tendency to linear force reduction response with an increasing time after reperfusion that may indicate the

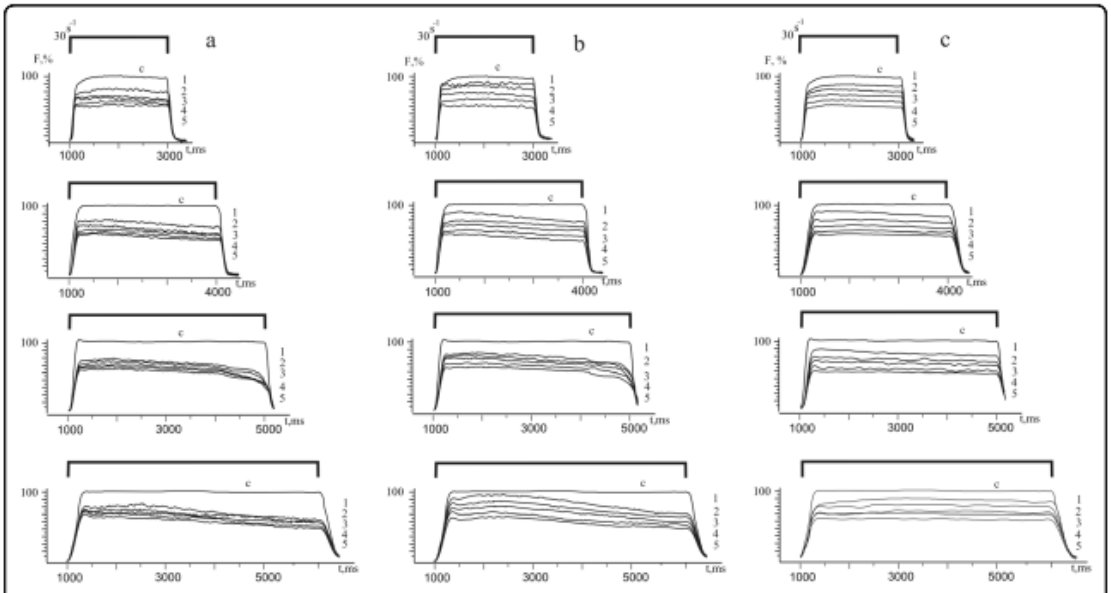

Fig. 1 Force generation curves (F, %) of the ischemic *soleus muscle*: **a** control (without affecting C_{60}FAS; $n = 10$); **b** intravenous injection of C_{60}FAS (dose 1 mg/kg; $n = 10$); **c** intramuscular injection of C_{60}FAS (dose 1 mg/kg; $n = 10$). *1, 2, 3, 4, 5* hours after reperfusion of the muscle

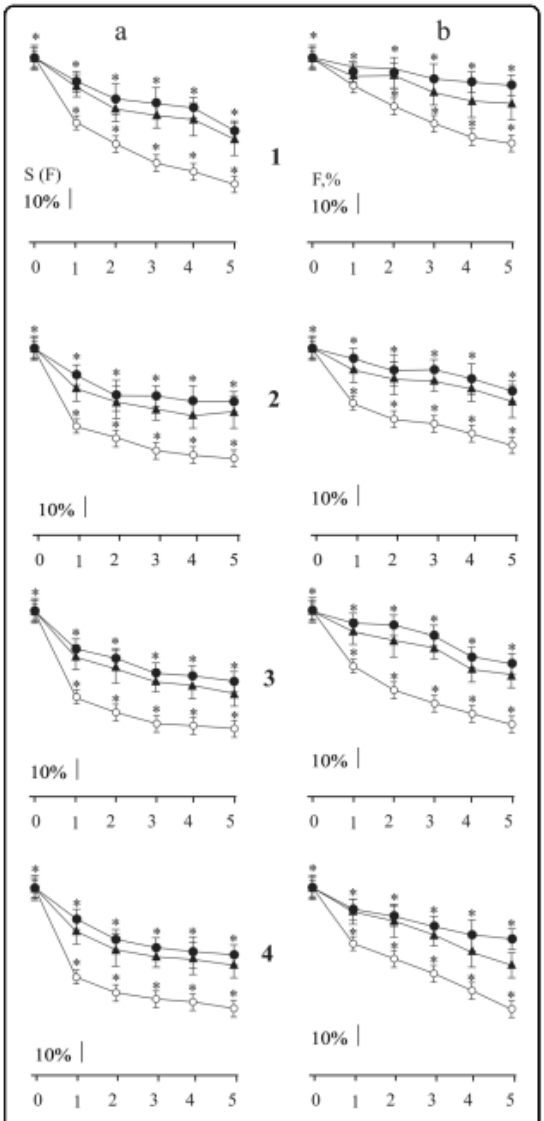

Fig. 2 Change of the integrated power S(F) (**a**) and maximal force of contraction (F) (**b**) (as a percentage of control values adopted by 100%) of the ischemic *soleus muscle* at different duration of modulated stimulation: *1, 2, 3, 4* stimulation *2, 3, 4, 5* s, respectively; (○) control (without affecting C_{60}FAS; $n = 10$); (▲) intravenous administration of C_{60}FAS (dose 1 mg/kg; $n = 10$); (●) intramuscular administration of C_{60}FAS (dose 1 mg/kg; $n = 10$). *0, 1, 2, 3, 4, 5* hours after reperfusion muscle. *$p < 0.05$

development of muscle fatigue. But unlike the fatigue process starting from the 2nd day of the experiment, the force curves contain the pronounced fluctuation components. If the power drop is caused by reduction of molecular generators of power, i.e., by reduction of working cross-bridges; then, in the case of fluctuation contractions, the damages should take place in almost all contractile components of the muscle cells. So, in this case, one can speak only about relatively similarity of force responses during fatigue and induced ischemia just in the early stages of pathological process. The significant dependence of dynamic characteristics of contraction on the activity of the main types of proprioceptors significantly complicates the control of the motor activity of the damaged muscle from the central nervous system (CNS) in case of uncontrolled fluctuation responses of ischemic-damaged muscle even on a simple stimulus signal. Elimination of these vibration components of muscle contraction with action of C_{60}FAS (regardless of the therapeutic administration method) is a very important feature of its protective effect (Fig. 3).

With using modulated stimulation, the quantitative and qualitative differences in the contraction of ischemic rat's *soleus muscle* in control and with C_{60}FAS were observed (Fig. 4). In control, the value of the maximum force and integrated power of muscle contraction decreased with an increasing time after reperfusion as well as the duration of stimulation (Fig. 4). Therapeutic administration of C_{60}FAS found the significant protective effect on contraction force characteristics that were studied as follows: the most pronounced protective effect was observed on the 5th day after ischemia and at maximum 5 s of stimulation; C_{60}FAS protective effect on the maximum force response was 30–35%, and the integrated power—over 50% compared to control. In this case, the difference between protective effects of C_{60}FAS depending on the method of administration was observed. Thus, intramuscular injection of C_{60}FAS showed 10–15% more protective effect on muscle force response in comparing with the intravenous administration of C_{60}FAS.

Differences in increasing force and integrated power of ischemic-injured muscles during intravenous and intramuscular administration of C_{60}FAS (Figs. 1, 2, 3, and 4) indicate the complexity of the molecular mechanisms of muscle contraction, which are probably different in implementing antioxidant properties of C_{60} fullerenes. Obviously, at the therapeutic injection of C_{60}FAS directly into the damaged muscle, the concentration of C_{60} fullerenes are much higher than in the area of inflammation compared with intravenous administration of C_{60}FAS. Thus, it can be argued on the realization of concentration dependence of the protective effect of C_{60} fullerenes on the maximum force contraction and integrated power of the ischemic injured muscles.

Observed high correlation between the duration of ischemia and muscle fiber survival [4] can be one of the main factors reducing the maximum force response with time increasing after ischemia not only due to the decrease of muscle fibers survival but also due to the increased rigidity of the muscle (due to an increase of its

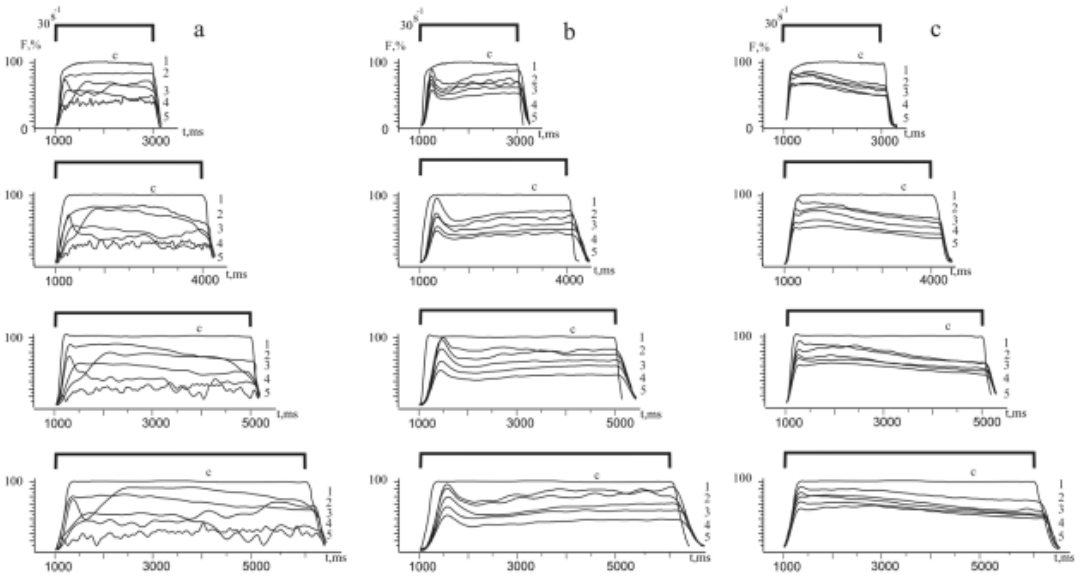

Fig. 3 Force generation curves (F, %) of the ischemic *soleus muscle*: **a** control (without affecting C_{60}FAS; $n = 10$); **b** intravenous injection of C_{60}FAS (dose 1 mg/kg; $n = 10$); **c** intramuscular injection of C_{60}FAS (dose 1 mg/kg; $n = 10$). *1, 2, 3, 4, 5* days after reperfusion of the muscle

collagen structures). After 3 h of ischemia, the muscle necrotic changes and nervous degradation occur. The amount of muscle tissue necrosis may be up to 60% [27]. In this case, the therapeutic action of C_{60}FAS will not have a positive effect. Thus, C_{60}FAS, use as a therapeutic agent for ischemic muscle damage, will have a pronounced beneficial effect mainly on the early stages of this disease.

According to modern theories of motor control in the development of muscle pathologies, CNS organizes the limb movements so as to reduce the number of degrees of freedom, which correspond to movements of the individual segments. The reason for this decrease is the synergies (involving functional activity of intact or partially damaged muscle fibers), which leads to complications of central management program movements, thus compromising control over the implementation of purposeful movements [28]. Because the structure of the dynamic component of stimulation (ratio of its amplitude to duration) determines the speed and range of motion, the changing nature of efferent activity realization of ischemic muscle results in errors in the positioning accuracy of the joint. By performing even simple movements, there is a possibility to establish causal links between the mechanical activity of ischemic-injured muscles of the joint and key dynamic parameters of movement. The accuracy of this analysis rises via detailed study of before tetanic areas of muscle contraction with simultaneous control of mechanical movement parameters [29]. Therefore, the studying changes in the dynamics of ischemic-damaged muscle contraction on before tetanic areas allow to detect the level of muscle damage and effectiveness of therapeutic action of C_{60}FAS.

Figure 5 shows changes in achieving speed of maximum force response of the ischemic muscle in dependence of time after ischemia: in the first 5 h and next 5 days after reperfusion. In control, after 1 h reperfusion, the reduction of maximum force and an increase of time to achieve it are observed. The therapeutic application of C_{60}FAS essentially adjusts the dynamics of the force curves: a clear separation of dynamic and stationary parts of the contraction occurs. It should be noted that this effect is independent of the manner of C_{60}FAS administration. A more pronounced effect of C_{60}FAS on before tetanic area muscle contraction and less on the maximum force response (Fig. 5), in our opinion, is connected with the beginning of irreversible pathological changes in the generation of force contraction on the most vulnerable before tetanic areas in the early stages of ischemic lesion of muscle cells. This is due to the uneven destruction of different molecular components of the contractile apparatus of muscle, which are activated in different phases of contraction and therefore face different impact of C_{60}FAS. However, even a slight protective effect of C_{60}FAS on tetanic areas of muscle contraction is essential in the first hours after launch of ischemic cascade of pathological processes and C_{60} fullerenes have to slow down their, particularly, neutralizing free radicals in early ischemic injury muscle.

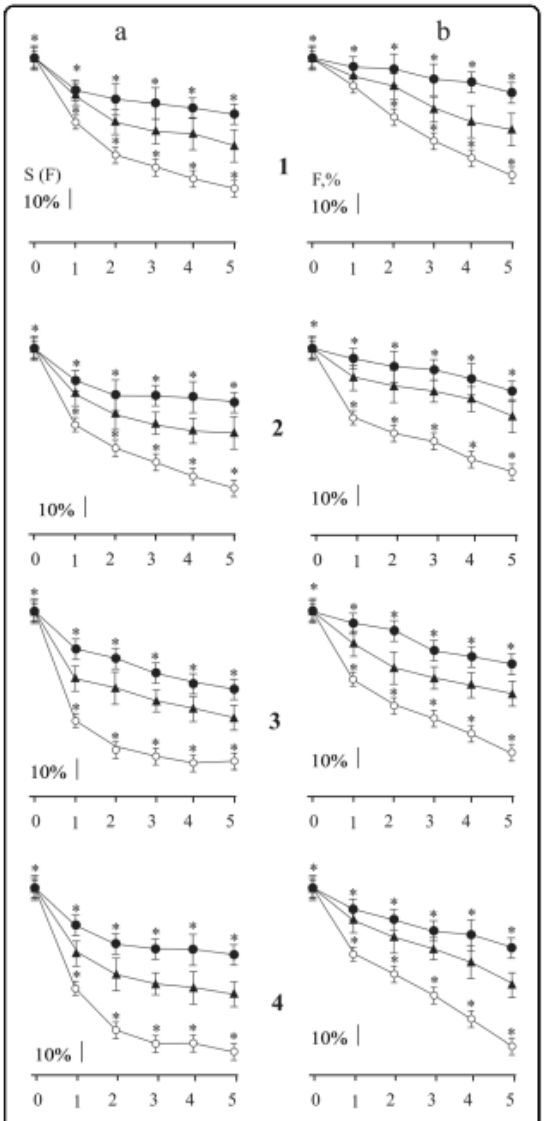

Fig. 4 Change of the integrated power S(F) **(a)** and maximal force of contraction (F) **(b)** (as a percentage of control values adopted by 100%) of the ischemic *soleus muscle* at different duration of modulated stimulation: 1, *2, 3, 4* stimulation *2, 3, 4, 5* s, respectively; (○) control (without affecting C_{60}FAS; $n = 10$); (▲) intravenous administration of C_{60}FAS (dose 1 mg/kg; $n = 10$); (●) intramuscular administration of C_{60}FAS (dose 1 mg/kg; $n = 10$). *0, 1, 2, 3, 4*, 5 days after reperfusion of the muscle. *$p < 0.05$

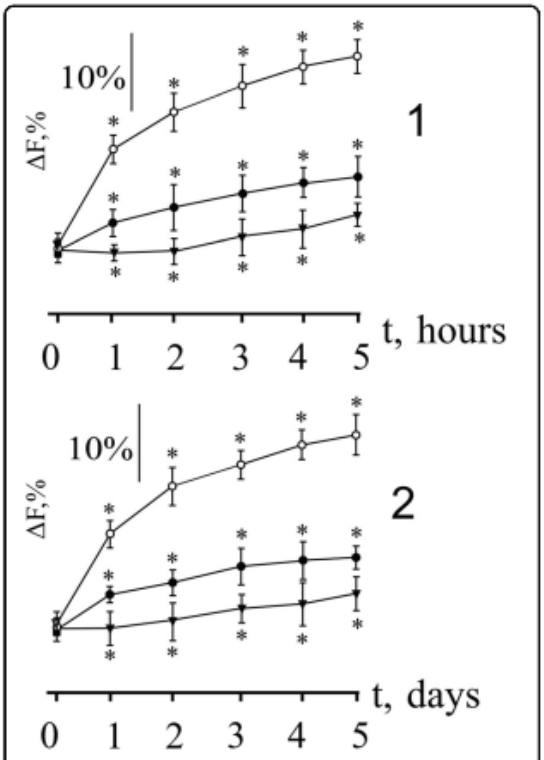

Fig. 5 Change of speed attaining maximum force response (ΔF, %) of the ischemic *soleus muscle*: (○) control (without affecting C_{60}FAS; $n = 10$), (▲) intravenous injection of C_{60}FAS (dose 1 mg/kg; $n = 10$), (●) intramuscular injection of C_{60}FAS (dose 1 mg/kg; $n = 10$). *1* and *2* hours and days, respectively, after reperfusion of the muscle. *$p < 0.05$

Research of speed change achieving maximum level of force within 5 days in control (Fig. 5) found a direct relationship between the speed reduction and time after reperfusion. Therapeutic administration of C_{60}FAS significantly corrected this parameter: its reduction after the 1st day after ischemia was not changed significantly over the next 4 days of experiment. Intravenous administration of C_{60}FAS demonstrated better protective effect (on 10%) compared to intramuscular administration of C_{60}FAS.

Biochemical Study

Most characteristic biochemical compounds which, on the one hand, easily identified in clinical conditions and, on the other hand, the content of which changes significantly upward in patients with ischemic injuries, are CPK and LDH [30].

CPK is contained in high concentration in the skeletal muscles, and body consumes it rapidly by increasing physical activity. If the damage of myocytes happens, CPK diffuses from them, thus increasing its activity in the blood. Therefore, determining the activity of CPK in the blood is a sensitive diagnostic test for the manifestation of ischemic damage in the muscle tissue [31].

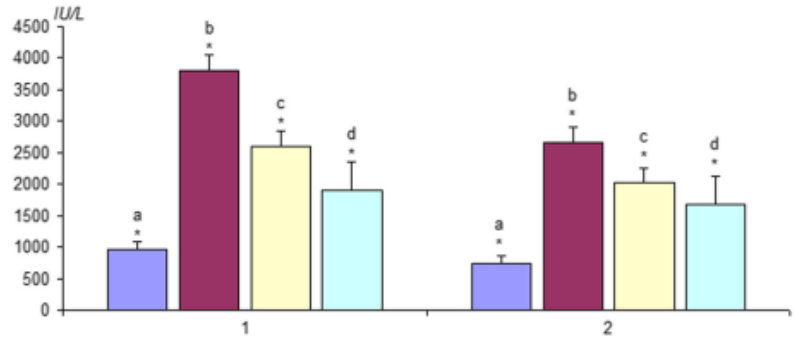

Fig. 6 Change in the content of creatine phosphokinase (1) and lactate dehydrogenase (2) in the ischemic *soleus muscle* 5 h after reperfusion: **a** intact animals ($n = 10$), **b** control (without affecting C_{60}FAS; $n = 10$); **c** intravenous injection of C_{60}FAS (dose 1 mg/kg; $n = 10$); **d** intramuscular injection of C_{60}FAS (dose 1 mg/kg; $n = 10$). $^*p < 0.05$

LDH participates in the processes of oxidation of glucose and the formation of lactic acid. It is contained in almost all organs and tissues of the human, especially a lot of it in the muscles. In the conditions of hypoxia, LDH causes a feeling of muscle fatigue and breaks the process of tissue breathing. Blood tests for CPK and LDH used in the clinic for the rapid identification of diseases associated with ischemic injuries of the muscular system [32]. Determination of the levels of these enzymes in the blood of tested animals showed accurate tendency to their reduction after therapeutic administration of C_{60}FAS after the first 5 h (Fig. 6) and 5 days after ischemia (Fig. 7).

Considering that at ischemic injury of the skeletal muscle, the reactive oxygen species (ROS) are of the most destructive danger, the use of C_{60} fullerenes as powerful antioxidants should significantly improve muscle tolerance to ischemia and expedite postoperative recovery [33].

The observed effects above may be related to the fact that 2 h ischemia-reperfusion of the *soleus muscle* significantly reduces the concentration of ATP with significant increase in lactate. It is known that, for a 3 h ischemia, ATP depletion is about 95%, and glycogen depletion is 88% [6, 7]. In addition, a large number of high-energy phosphates are spent by the damaged muscle cell to maintain hemostasis and, as a result, the metabolic disorder leads to greater muscle fatigue. At the same time, literature data indicate that ROS (for example, superoxide anion and hydroxyl radical) are a major pathogenic factor in ischemia-reperfusion tissue damage. ROS initiate the lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate dehydrogenase, ATPase inhibition activity, inactivation of membrane sodium channels, etc. It was shown that modified C_{60} fullerenes can be considered as a powerful ROS absorber of ischemia-reperfusion-induced injury of small intestine [34].

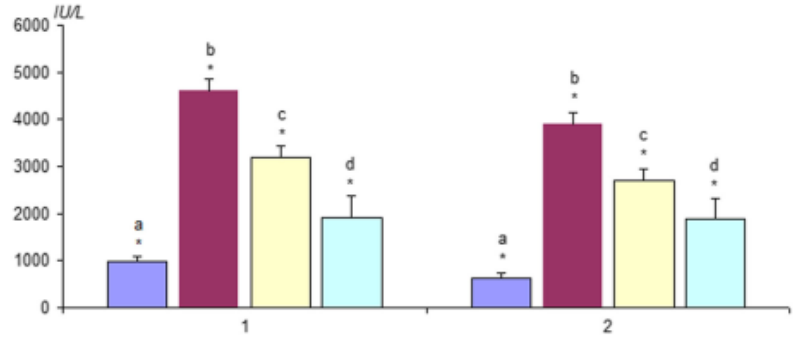

Fig. 7 Change in the content of creatine phosphokinase (1) and lactate dehydrogenase (2) in the ischemic *soleus muscle* after 5 days after reperfusion: **a** intact animals ($n = 10$), **b** control (without affecting C_{60}FAS; $n = 10$), **c** intravenous injection of C_{60}FAS (dose 1 mg/kg; $n = 10$), **d** intramuscular injection of C_{60}FAS (dose 1 mg/kg; $n = 10$). $^*p < 0.05$

Also, the ability of C_{60} fullerene derivatives to reduce the ischemia-reperfusion lung injury was demonstrated [35, 36]. In this regard, the protective effect of C_{60}FAS on the fatigue processes of the ischemic-damaged muscle can be directly linked to the strong antioxidant properties of pristine C_{60} fullerenes.

Conclusions

The results of this study can be united in the following paragraphs:

1. A pronounced protective effect of C_{60}FAS on the contractile dynamics of *muscle soleus* ischemic injury was reliably established.
2. It was shown that intravenous and intramuscular injections of C_{60}FAS have different therapeutic effects: the intravenous injection of C_{60}FAS is optimal for correction of speed macroparameters of contraction at ischemic muscle damage; the intramuscular injection of C_{60}FAS demonstrates more pronounced protective effect in movements associated with the generation of maximum force responses or prolonged contractions caused by increasing levels of muscle fatigue. It must be emphasized that protective effect of C_{60}FAS is also important to correct the accuracy of the joint position of the injured limb, since for precision positioning and fine motor skills of limbs, extremely important is the ability to hold the tetanic contraction regime by the antagonist muscles, the implementation of which is lost during ischemic pathology.
3. The use of biocompatible water-soluble pristine C_{60} fullerenes considering prominent antioxidant properties and lack of data of acute and chronic toxicity open new possibilities in the therapy and prevention of ischemic pathologies.

Abbreviations

C_{60}FAS: C_{60} fullerene aqueous colloid solution; CNS: Central nervous system; CPK: Creatine phosphokinase; LDH: Lactate dehydrogenase; ROS: Reactive oxygen species

Authors' Contributions

All authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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C_{60} fullerene as promising therapeutic agent for correcting and preventing skeletal muscle fatigue

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Abstract

Background: Bioactive soluble carbon nanostructures, such as the C_{60} fullerene can bond with up to six electrons, thus serving by a powerful scavenger of reactive oxygen species similarly to many natural antioxidants, widely used to decrease the muscle fatigue effects. The aim of the study is to define action of the pristine C_{60} fullerene aqueous colloid solution (C_{60}FAS), on the post-fatigue recovering of *m. triceps surae* in anaesthetized rats.

Results: During fatigue development, we observed decrease in the muscle effort level before C_{60}FAS administration. After the application of C_{60}FAS, a slower effort decrease, followed by the prolonged retention of a certain level, was recorded. An analysis of the metabolic process changes accompanying muscle fatigue showed an increase in the oxidative stress markers H_2O_2 (hydrogen peroxide) and *TBARS* (thiobarbituric acid reactive substances) in relation to the intact muscles. After C_{60}FAS administration, the *TBARS* content and H_2O_2 level were decreased. The endogenous antioxidant system demonstrated a similar effect because the *GSH* (reduced glutathione) in the muscles and the *CAT* (catalase) enzyme activity were increased during fatigue.

Conclusions: C_{60}FAS leads to reduction in the recovery time of the muscle contraction force and to increase in the time of active muscle functioning before appearance of steady fatigue effects. Therefore, it is possible that C_{60}FAS affects the prooxidant-antioxidant muscle tissue homeostasis, subsequently increasing muscle endurance.

Keywords: C_{60} fullerene, Skeletal muscles fatigue, Electrical stimulation, Oxidative stress markers, Antioxidant system

Background

Skeletal muscle fatigue is the defence mechanism against overload and leads to the development of painful muscle sensitivity [1–3]. Muscle fatigue develops after physical activities of varying intensities and often leads to acute pain, which can then lead to various chronic disease states [4, 5]. Muscle fatigue is a result of the products of incomplete oxygen oxidation, such as reactive oxygen species (ROS), including peroxides, free radicals, and oxygen ions [6]. During the course of lipid peroxidation, unsaturated fatty acids are formed from various fatty acid derivatives and metabolites, such as malondialdehyde and hydroperoxide fatty acid [7]. The excessive accumulation of ROS (oxidative stress) can lead to significant functional changes due to damage to different cell components [8]. An example is the lipid peroxidation of biological membranes, which promotes the disruption of their structure and increases their permeability [9]. Cell protection against such damage is provided by the antioxidant system. Mach et al. [10] used pycnogenol as an antioxidant, and its use is accompanied by an increase in the levels of both oxidized and reduced NAD^+ in the serum, as well as increased muscle strength. In studies of muscle fatigue, endogenous antioxidants, such as an N-acetylcysteine [11] and β-alanine [12], are widely used and speed up the muscle recovery process after fatigue.

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In this context, bioactive soluble carbon nanostructures, such as the pristine C_{60} fullerenes, may be considered potential antioxidants [13]. C_{60} fullerene easily bonds with up to six electrons, can serve as a powerful scavenger of ROS [13–15], and is superior to the majority of natural antioxidants, including vitamins C and E and carotenoids, in regard to its antioxidant capacity. As a result, it prevents oxidative stress dissemination in thymocytes [16] and shows a protective effect following the ischemia–reperfusion injury of skeletal muscle [17]. Additionally, water-soluble pristine C_{60} fullerenes can penetrate through the plasma membrane of cells [18, 19]. Therefore, the use of C_{60} fullerenes may have a powerful antioxidant effect on the contractile apparatus of striated muscle, thereby facilitating its functional recovery after experimentally induced fatigue.

The aim of this study was to investigate the effect of water-soluble pristine C_{60} fullerenes on the recovery dynamics of the contractile properties of rat *m. triceps surae* (*TS*) after the development muscle fatigue under conditions of long-term activation.

Methods

Material preparation and characterization

A highly stable reproducible pristine C_{60} fullerene aqueous colloid solution (C_{60}FAS) at a concentration of 0.15 mg/ml was prepared according to a previous protocol [20, 21]. Briefly, for the preparation of C_{60}FAS we used a saturated solution of pure C_{60} fullerene (purity >99.99%) in toluene with a C_{60} 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 ultrasonic bath. The procedure was continued until the toluene had completely evaporated and the water phase became yellow colored. Filtration of the aqueous solution allowed to separate the product from undissolved C_{60} fullerenes. The pore size of the filter during the filtration of the aqueous solution was smaller than 2 μm (Typ Whatmann 602 h1/2). The purity of prepared C_{60}FAS (i.e., the presence/absence of any residual impurities, for example carbon black, toluene phase) was determined by HPLC and GC/MS analysis. The maximal concentration of C_{60} fullerenes in water 0.15 mg/ml was obtained by this method.

The state of C_{60} fullerenes in aqueous solution was monitored using atomic force microscopy (AFM). Under AFM analysis, the sample was deposited onto a cleaved mica substrate (V-1 Grade, SPI Supplies) by precipitation from an aqueous solution droplet. Sample visualization was performed in semi-contact (tapping) mode (Fig. 1a, b). AFM measurements were performed after the complete evaporation of the solvent.

Small-angle neutron scattering (SANS) measurements (Fig. 1c) were carried out at the YuMO small-angle diffractometer at the IBR-2 pulsed reactor (JINR, Dubna, Russia) in the time-of-flight mode with the two-detector setup [22]. Treatment of the raw data was performed by the SAS program [23].

Procedure and experimental groups

Male Wistar rats, weighing 280–350 g, were used in the study. The use of the animals was approved by the Ethics Committee of the Institute and performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

The animals were divided into 4 groups. In the experiments, the *m. triceps surae* fatigue was induced by electrical stimulation of *n. tibialis*. Saline solution (group 1, n = 6) or C_{60}FAS (F-injection) 0.1–0.15 mg/kg (group 2, n = 6) was administered into the left *TS* after the development of fatigue. Then, fatigue of the right *TS* was induced. The data obtained from the ipsilateral (left) side were considered to be the control values vs. those obtained from the contralateral side. The dose range of 0.1–0.15 mg/kg C_{60}FAS does not present any acute or subacute toxicity in rats [13]. The rats of group 3 (n = 6; animals with fatigue of both *TS* without any injections) and group 4 (n = 6; intact animals) were used only for biochemical studies. After the experiment, the *TS* of all animals in all groups were removed for biochemical analysis.

It is important to note that a dose of 0.1–0.15 mg/kg C_{60}FAS applied in our experiments does not present any acute or subacute toxicity in animals: it was significantly lower than the maximum tolerated dose of C_{60} fullerene, which was found to be 5 g/kg both for oral or intraperitoneal administration to rats [13]. No toxic effects or death have been fixed under the action of C_{60} fullerenes after their oral administration to rats in total dosage of 2 g/kg for 14 days [24]. Finally, it was shown [13] that water-soluble C_{60} fullerenes administered intraperitoneally to rats (0.5 mg/kg) were subjected to clearance from the organism within 2–4 days.

The animals in groups 1 and 2 were anaesthetized with ketamine (100 mg/kg "Pfizer", USA) combined with xylazine (10 mg/kg, "Interchemie", Holland), tracheostomized and artificially ventilated (out of necessity). The left and right *TS* muscles were separated from the surrounding tissue, and their tendons were detached at the distal insertions. The *n. tibialis* was separated from the tissue and cut proximally, and all branches of the nerve, except those innervating the *TS*, were cut. This nerve was mounted on a bipolar platinum wire electrode for electrical stimulation. The hindlimb muscles and nerves were covered with paraffin oil in a pool formed from skin flaps.

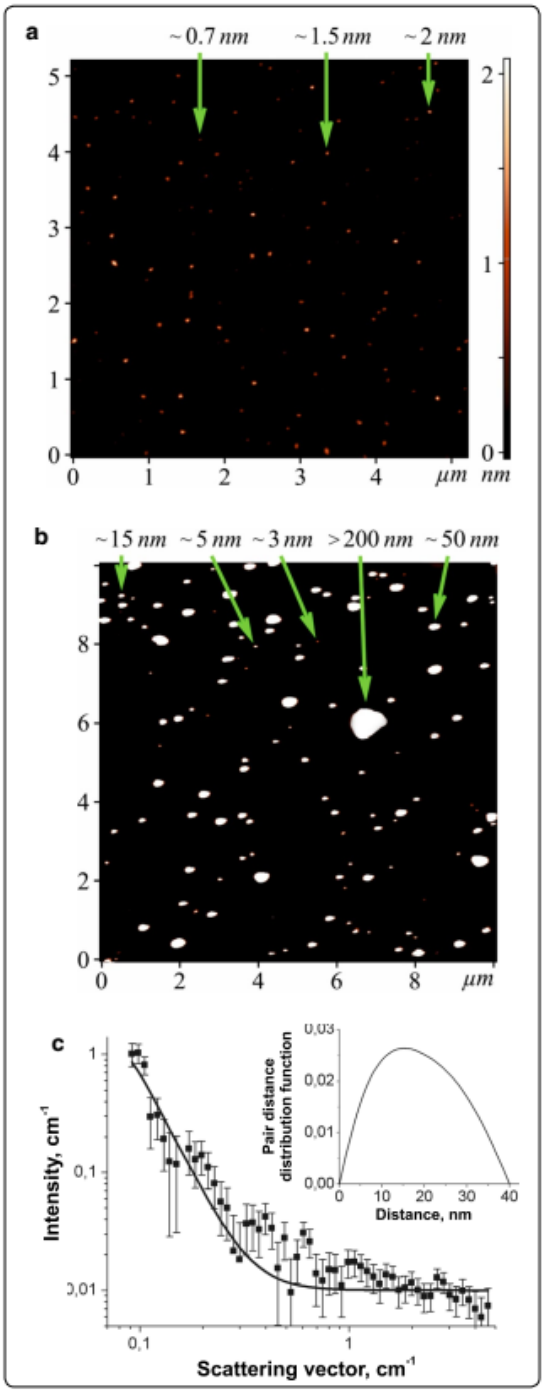

Fig. 1 AFM images (tapping mode) of C_{60} fullerene particles on the mica surface, which were precipitated from C_{60}FAS with an initial concentration of 0.15 mg/ml (**a**, **b**). *Arrows* indicate the height of the individual particles. Experimental SANS curve (*points*) for C_{60}FAS (0.15 mg/ml). Solid lines correspond to the model curve obtained by the IFT procedure. Insert: the pair distance distribution function as a result of the IFT procedure for scattering from C_{60} fullerene nanoparticles present in the C_{60}FAS (**c**)

The *TS* muscle was connected via the Achilles tendon to the servo-control muscle puller. The muscle tension was measured by semi-conductor strain gauge resistors glued on a stiff steel beam mounted on the moving part of a linear motor.

To induce muscle fatigue, 1–3 (30 min duration) series intermittent high-frequency electrical stimulation was used (Fig. 2a), separated by rest intervals of 10–20 min. Each series consisted of trains of 0.2-ms rectangular pulses at a rate of 40/s at 12.4 s duration and separated by 5 s intervals of rest (Fig. 2b). The stimulus current was set to 1.3–1.4 times the motor threshold. Note, if muscle fatigue developed in less than 30 min, stimulation was interrupted (it was predicted that fatigue development occurred when there was a muscle force decrease of more than 50% of the initial data). After the end of the 12.4-s-stimulation, the muscle was stretched, and the change in length had a bell-shaped form (one period of 4 Hz sinusoidal signal with corresponding phase locking) of 3.5 mm amplitude and 2 s duration (Fig. 2b; bottom row). The muscle reaction to the stretches appeared as a tension increase after continuous stimulation. These stretches were applied before the post-stimulation twitches to remove, or at least diminish, the after-effects remaining from the continuous stimulation [1]. The signals (stimulus pulses, muscle tension and other) were sampled via DAC-ADC device (CED Power 1401).

Biochemical experiment

For biochemical analysis, the excised *m. triceps surae* (soleus and gastrocnemius) were rapidly dissected, free of fat and tendon, divided into several portions and stored in liquid N_2. For reduced glutathione (*GSH*) analysis, tissue samples were transferred into a medium containing 1 N perchloric acid (1:10 w/v) and homogenized with a motor-driven Potter–Elvehjem glass homogenizer. The resultant homogenate was centrifuged at 10,000*g* for 10 min (4 °C). The *GSH* content was spectrophotometrically measured [25]. For the enzyme activity assays and H_2O_2 and lipid peroxidation assays, the muscle samples were thawed and homogenized in 50 mM

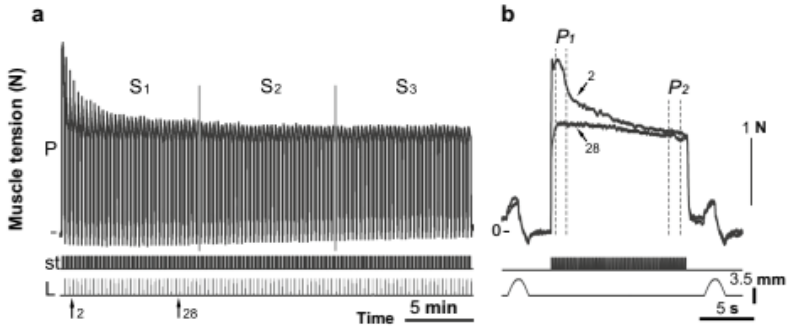

Fig. 2 Strength of the contralateral (*right*) *m. triceps surae* (*TS*) contraction during the 2nd series of 30-min intermittent stimulations of the *n. tibialis* at 42 min after C_{60}FAS administration into the left *TS* (**a**). The superposition of individual tetanic contractions 2 and 28 are presented at the higher time scale (**b**). *P* muscle force, *st* stimulation mark, *L* muscle length (mm); S_1, S_2 *and* S_3 equal parts of the stimulus series, used for the quantitative analysis of data; P_1 and P_2 sites at the beginning and at the end of tetanic contraction

phosphate buffer with 2 mM EDTA (pH 7.4) at 4 °C (1:9 w/v). Homogenates were then centrifuged for 15 min at 15,000*g* (4 °C), and the post mitochondrial supernatant was stored at −70 °C.

Oxidative damage in the tissue was measured using the thiobarbituric acid reactive substances (*TBARS*) assay. *TBARS* were isolated by boiling tissue homogenates for 15 min at 100 °C with thiobarbituric acid reagent (0.5% 2-thiobarbituric acid/10% trichloroacetic acid/0.63 M/dm^3 hydrochloric acid) and measuring the absorbance at 532 nm. The results are expressed as nM *TBARS*/mg protein, using $\varepsilon = 1.56 \times 10^5\ dm^3/M^1/cm^1$ [26].

The H_2O_2 concentration in the tissue homogenates was measured using the FOX method, which is based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange (o-cresolsulphonephthalein 3′,3″-bis[methylimino] diacetic acid, sodium salt). This method is extremely sensitive and is used to measure low levels of water-soluble hydroperoxide present in the aqueous phase. To determine the H_2O_2 concentration, 500 µl of the incubation medium was added to 500 µl of assay reagent (500 µM ammonium ferrous sulphate, 50 mM H_2SO_4, 200 µM xylenol orange, and 200 mM sorbitol). The absorbance of the Fe^{3+}-xylenol orange complex (A_{560}) was detected after 45 min. Standard curves of H_2O_2 were obtained for each independent experiment by adding variable amounts of H_2O_2 to 500 µl of basal medium mixed with 500 µl of assay reagent. Data were normalized and expressed as µM H_2O_2 per mg protein [27].

Catalase activity was measured by the decomposition of hydrogen peroxide, determined by a decrease in the absorbance at 240 nm [28].

GSH was determined using Ellman's reagent. One millilitre of supernatant was treated with 0.5 ml of Ellman's reagent (5.5′-dithio-bis-nitrobenzoic acid in abs. ethanol) and 0.4 M Tris HCl buffer with 2 mM EDTA, pH 8.9. The absorbance was read at 412 nm in a spectrophotometer [25].

The protein concentration was estimated using the method of Bradford with bovine serum albumin as a standard. All chemicals were purchased from Sigma, Fluka and Merck and were of the highest purity.

Data analysis

In the electrophysiological part of the study, each stimulation series (30 min) was divided into three equal portions (Fig. 2a), which were averaged (maximum 33 stimulation in one portion). The average value of the first portion was set to 100%, and the other series were normalized in relation to this (for each hindlimb). The peak amplitudes of the front (P_1) and rear of the front (P_2) (maxima amplitudes at the site, duration of 1 s, Fig. 2b) of the muscle strength of each single series (12.4 s) were identified and the difference between P_1 and P_2 (ΔP) was calculated. This difference determines the dynamic component of the muscle force decrease in a short period of continuous stimulation. Mean values (mean ± SD) of the *TS* muscle strength before and after F-injection were compared using a two-way statistical analysis of variance (ANOVA). The factors of variation included two conditions, time and the effects of the C_{60}FAS. A Bonferroni post hoc analysis was used to determine the differences between groups. The level of significance was set at $p < 0.001$.

Biochemical data are expressed as the means ± 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.

Results

Analysis of AFM and SANS data

Because the C_{60} fullerene particle size directly correlates with their biodistribution and toxicity [29, 30], the AFM and SANS studies were performed.

The AFM images (Fig. 1a, b) clearly demonstrate randomly arranged, individual C_{60} fullerenes (0.7 nm in diameter) and their bulk clusters with a height of 1.5–200 nm. At the same time, some individual C_{60} fullerene aggregates with a height of >200 nm are also seen in the AFM image (Fig. 1b). The results obtained are consistent with the theoretical calculations and experimental measurements [20, 21, 31, 32] and demonstrate the polydispersity of the C_{60}FAS used in our study.

Experimental SANS curve for C_{60}FAS is shown in Fig. 1c. The scattering curve of C_{60}FAS is well described by the form-factor of polydisperse spherical particles. The mean radius of gyration of the particle cross section, R_g, and pair distance distribution function, P(r), were found by using indirect Fourier transformation (IFT) approach [33]. We can calculate the radius of particles, R, present in the C_{60}FAS according to well-known equation $R_g^2 = 0.6R^2$ assuming of homogeneous and spherical of C_{60} fullerene clusters. This conclusion follows from previous experimental data [20, 21] and the estimates of the average cluster density according to the contrast-variation experiments [31, 32, 34]. The data given by this procedure indicate that C_{60}FAS consists of C_{60} fullerene sphere-like nanoparticles with an average size of ~56 nm that is in a good agreement with above AFM data.

It is known [35, 36] that the permeability and cytochemical behavior of nanoparticles strongly depend on their size and, correspondingly, mass (number) distribution. In this regard, our previous studies [16, 18, 19, 29] clear demonstrate that the used C_{60} fullerene nanoparticles can effectively penetrate through the plasma membrane of cells by passive diffusion or endocytosis (depending on the size) and do not exhibit cytotoxic effects.

Electrophysiological experiments

Changes in the *TS* force reaction under fatigue conditions due to prolonged high frequency stimulation (30 min, 40/s) of the *n. tibialis* for animal groups 1 (before and after administration of saline solution) and 2 (before the application of C_{60}FAS) did not significantly differ. The analysis was performed by determining the force level at the beginning (P_1) and end (P_2) of single tetanic contractions and the difference between these values (ΔP), which determines the dynamic component of the force decrease during a short period of continuous stimulation (Fig. 2b). The muscle was considered tired if the amplitude of the single tetanic contractions decreased by more than 50% relative to the initial level. When muscle fatigue was reached, the stimulation was stopped and followed by a 10–20 min rest period. Therefore, in the case of one animal, as a result of muscle fatigue stimulation of the left *TS*, a 50% fatigue level was reached in approximately 12 min; during the next 4 min of stimulation, it continued to decrease [Fig. 3a (I_L), b(I_L)]. After 10 min of rest, a single tetanic contraction force was slightly restored, but it did not reach the initial muscle activity level and continued to decrease rapidly [Fig. 3a (II_L), b(II_L)]. In this case, there was also a simultaneous decrease in the dynamic component of the force drop ΔP. Note that the dynamic component was the most highly expressed at the beginning of the first experimental series and that the P_1 amplitude was higher relative to the P_2 amplitude [Fig. 3b (II_L)]. After tetanic contractions for 1.5–2 min, difference between amplitudes P_1 and P_2 was reduced to zero, with moderate variations both in one and the opposite direction over the additional fatigue stimulation period. Simultaneously with the decrease in ΔP values, there was a constant decrease in the developed force. In the following stimulation series, after a period of rest, the initial amplitude of the dynamic component was usually decreased [Fig. 3b (II_L, III_L)].

When a predetermined level of muscle fatigue was reached, C_{60}FAS (0.1–0.15 mg/kg) was injected intramuscularly [at 45 min after the beginning of fatigue stimulation; Fig. 3a (I_L^F, II_L^F), b(I_L^F, II_L^F)]. At the same time, the dynamic changes in the muscle strength level in response to stimulation reflected the further development of fatigue, and the single contraction forces were reduced rapidly [Fig. 3b(I_L^F)]. However, F-injection led to the gradual recovery of the isometric force levels (at 32 min after drug application; Fig. 3a [(II_L^F), b (II_L^F)]. The appearance of negative ΔP values (P_2 amplitude increase compared to P_1 amplitude) indicated the beginning of the recovery [Fig. 3b (II_L^F), 10th min]. In this series of stimulations, the level of the muscle contraction force was recovered to that developed during the initial stages of fatigue stimulation.

Power reaction of the right *TS* was significantly different from the left *TS*. Notably, the *TS* of the right limb was not previously fatigued before the F-injection (Fig. 3c, d). At 52 min after drug administration, a certain force muscle decrease was observed. In this case, the P_1 amplitude was higher than the P_2 amplitude, as indicated by the increase in ΔP values [Fig. 3c (I_R^F), d (I_R^F)]. However, at 6 min after the beginning of fatigue stimulation, the force developed by the muscle appeared at a certain stationary level, which was held during the experimental series. The difference between the P_1 and P_2 amplitudes disappeared (value of ΔP decreased to zero), which may indicate a constant force level at the time of loading. It

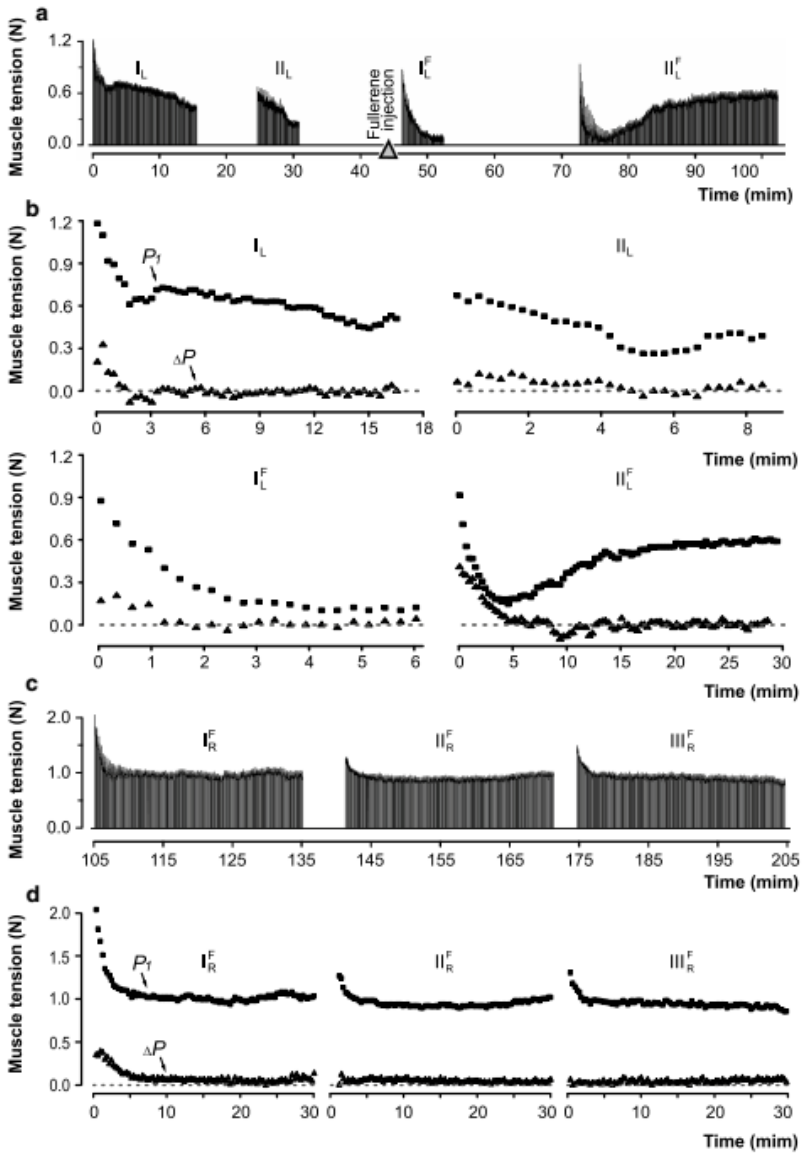

Fig. 3 Strength of the ipsi- and contralateral *m. triceps surae* (*TS*) contraction induced by electrical fatigue stimulation before and after C_{60}FAS injection into the *left TS*: **a**, **c** time protocol registrations of the *left* and *right TS* contraction, respectively (*triangles* indicate the moment the of the C_{60}FAS injection); **b**, **d** amplitude values of the muscle force (P_1) at the beginning of single tetanic contractions (*squares*) and ΔP (the difference between the force values at the beginning and at the end of muscle contraction; *triangles*). The rapid development of fatigue (**a**) (decrease in the muscle strength of more than 50%) led to shortening of the stimulation time (I–III). Designations for I–IV (**a**) and I–III at (**c**) correspond to recordings on (**b**) and (**d**), respectively. Indices: *L*, *R* left and right *TS*; *F* registration of muscle force after the administration of C_{60}FAS into the left *TS*

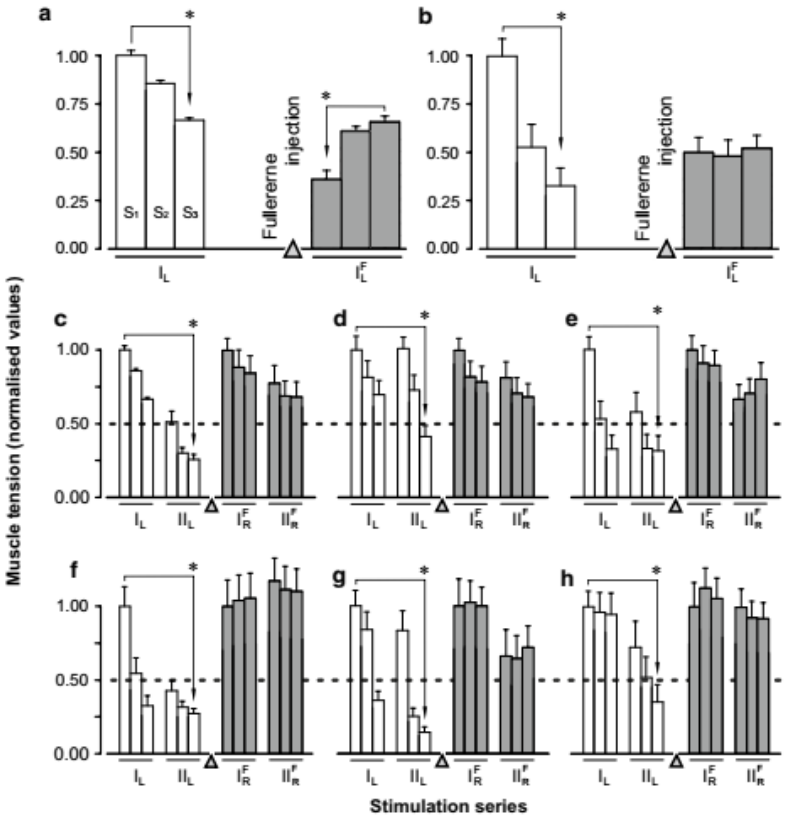

Fig. 4 Averaged characteristics (mean ± SD) of normalised (to average values S1) values of the muscle strength during different parts of the fatigue stimulation (S1, S2, and S3; Fig. 2) before and after (*white* and *grey bars*, respectively) C_{60}FAS administration into the left *m. triceps surae* (*TS*): **a**, **b** the results of two fatigue tests before and after C_{60}FAS administration into the left *TS*; **c**–**h** the results of six fatigue tests of the left *TS* (*open bars*) before C_{60}FAS administration, and right *TS* (*grey bars*) at 52 min after C_{60}FAS administration into the left *TS*. *Asterisks* significant differences ($p < 0.001$) between the muscle strength during time intervals S_1 and S_3 in one or more series of the stimulation. *I, II* successive series of fatigue stimulations (normalisation performed by S_1 in series I). Indices: *L, R* left and right *TS*; *F* registration of muscle force after the administration of C_{60}FAS into the left *TS*. *Triangle marks* the moment of C_{60}FAS injection

is significant that the muscle maintained the developed force level for an additional 1 h [Fig. 3c (II_R^F, III_R^F), d(II_R^F, III_R^F)]. For this muscle, the total time of the decrease of the isometric force contraction by 50% was 120 min after drug administration. For comparison, the control duration of the fatigue occurrence period was 42 min.

In Fig. 4a, b, a comparison of the force level changes developed by the left *TS* before (I_L) and after (I_L^F) F-injection in two different experiments is presented. The statistical analysis showed a significant ($p < 0.001$) force decrease during the series of fatigue stimulation before C_{60}FAS administration [Fig. 4a (I_L), b(I_L)]. After F-injection, a recovery of the muscle force for the first animal [Fig. 4a (I_L^F)] and its holding for the second animal [Fig. 4b (I_L^F)] was observed. At the end of the experimental series, the recovery of the active muscle force response was significant compared to that at the beginning and nearly reached the control values. The analysis showed a significant effect for the factors: drug administration (D) and time (T) after administration and their interaction. The corresponding results of this analysis were as follows: $F(D) = 2904.47$, $p(D) < 0.001$, $F(T) = 42.420$, $p(T) < 0.001$, $F(DxT) = 1350.58$, $p(DxT) < 0.001$ (first animal) and $F(D) = 122.80$, $p(D) < 0.001$, $F(T) = 1058.29$, $p(T) < 0.001$, $p(DxT) = 1287.35$, $p(DxT) < 0.001$ (second animal). The data obtained in all experiments (Fig. 4c–h) indicate that the decrease in the developed force after C_{60}FAS administration (I_L^F, II_R^F) was almost two times

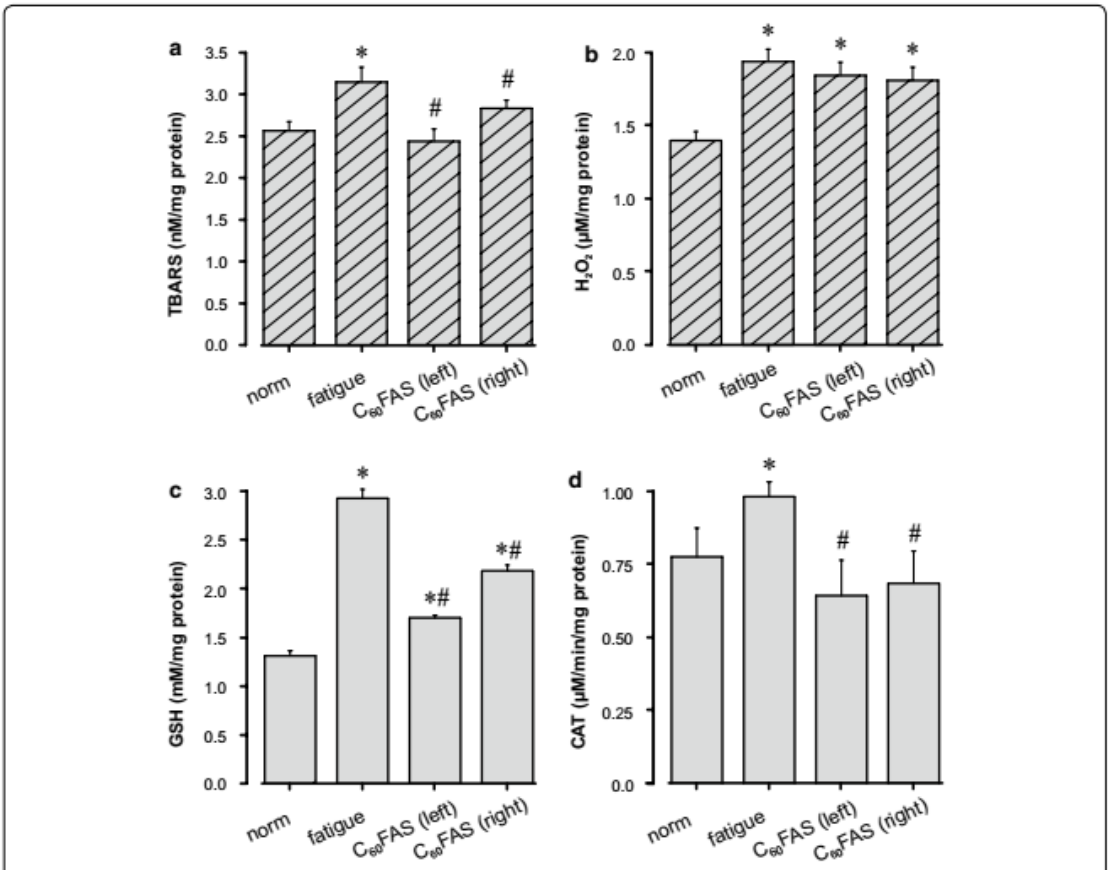

Fig. 5 Indicators of the prooxidant-antioxidant balance in the *m. triceps surae* (*TS*) of rats. The concentration of thiobarbituric acid reactive substances (*TBARS*) (**a**), hydrogen peroxide (H_2O_2) (**b**), glutathione (*GSH*) (**c**) and catalase (*CAT*) (**d**) are in intact animal muscles (norm), with the left fatigued TS (fatigue) and after C_{60}FAS administration ipsi- and contralaterally [C_{60}FAS (*left*) and C_{60}FAS (*right*), correspondingly]. Values are the mean ± SEM, n = 6. *p < 0.05 vs. "norm"; #p < 0.05 vs. "fatigue"

slower than in controls. The maximum significant reduction of the muscle force developed during the entire period of fatigue stimulation was 44% after drug administration, whereas in the control this was 85%. For all experimental animals, similar dynamics of the force level decrease in the control and its more gradual decrease after F-injection were observed.

Biochemical experiments

During long-term stimulation of the muscle, metabolic processes change and are a main factor of muscle fatigue. As a result of the fatigue test, the accumulation of lipid peroxidation secondary products and changes in the levels of antioxidants in the tissue of the fatigued muscle were determined. The data clearly demonstrate the increased level of peroxidation and oxidative stress marker *TBARS* and H_2O_2 after fatigue stimulation (Fig. 5a, b). This increase was significant in relation to the intact muscle ('*norm*') and was 23% ($p < 0.05$) for *TBARS* and 38% ($p < 0.05$) for H_2O_2. After C_{60}FAS administration into the left *TS*, the *TBARS* concentration was significantly reduced compared to fatigue as follows: 29% ($p < 0.05$) for the left *TS* and 12% ($p < 0.05$) for the right one. The H_2O_2 level decreases in comparison to the '*fatigue*' group (by 6% for the left *TS* and 7% for the right one), although the H_2O_2 level remained higher in relation to the intact group ($p < 0.05$). In turn, in response to such changes in the working muscle, an activation of endogenous antioxidants occurred. During fatigue stimulation, the amount of muscle *GSH* quantitatively increased more

than two-fold ($p < 0.05$) and the activity of the antiperoxide enzyme *CAT* also increased. After C_{60}FAS administration, the *GSH* and *CAT* activities were significantly decreased compared to the group *'fatigue'* by 41.8 and 15.4% for *GSH* and 53 and 43% for *CAT* ($p < 0.05$) for the left and right *TS*, respectively (Fig. 5c, d).

Discussion

In this study, we investigated changes in the contraction force of the rat *m. triceps surae* under fatigue development before and after C_{60}FAS administration. We did not use a level of stimulation above 40 Hz, and the rest period between the experimental series was 15–20 min [2]. This experimental approach allows us to analyse the nature of the muscle contraction force parameter changes under fatigue stimulation before C_{60}FAS application (into the left *TS*) and directly after F-injection. A marked decrease in the muscle effort level before C_{60}FAS administration (control) was observe in the all experiments both I_L and II_L stimulation series (Fig. 4a–h). It was the result of modified stimulation pattern action, which was due to the influence of the central and peripheral mechanisms of the development of skeletal muscle fatigue [2]. After intramuscular injection of the C_{60}FAS partial ipsilateral *TS* muscle recovery was registered in two rats. However, the main finding was observed after the application of C_{60}FAS. Not significant a slower effort decrease, followed by the prolonged retention of a certain level was recorded contralaterally in all animals. Decrease in the muscle contraction force was developed more slowly after C_{60}FAS administration compared to the control. It indicates a deceleration of the fatigue process, and the strength restraint at the constant level for a long time (120 min) indicates an increase in the muscle endurance during such conditions. The data obtained in this study indicate that after drug injection, the time for the *TS* force maximal level decrease to 44% was 120 min. At the same time in the control, the force level of this muscle during the same period decreased to 85%. We suppose, it was caused by antioxidant effects C_{60}FAS on the fatiguing muscle. The duration of the muscle recovery and its rest periods are also important factors for maintaining efficiency and the normal physiological state of the muscle during dynamic work execution [12]. The dynamic component of the single tetanic contraction is likely a reflection of the interaction of the efficiency of the initial increase of the fast motor unit contractile properties and processes of the fatigue strength reduction [37]. Thus, recovery of muscle strength after F-injection both for the preliminary tired and at fresh muscles indicate, that water-soluble pristine C_{60} fullerenes can penetrate through the plasma membrane of cells [18, 19] and render of powerful antioxidant effect on the contractile apparatus of striated muscle, thereby facilitating its functional recovery after experimentally induced fatigue.

Under a moderate external load on the muscle, metabolism occurs aerobically. In the actively contracted muscle, metabolism significantly increases, resulting in the accumulation of secondary oxidation products in muscle fibres, which leads to fatigue development [38]. These metabolic processes are a source of oxygen free radicals and contribute to the intensification of lipid peroxidation processes [39–41]. The presence of such metabolism products prevents the adequate implementation of muscle work and increases the duration of the recovery period. Strenuous exercise and endurance training cause oxidative stress in skeletal muscle and can therefore alter the prooxidant-antioxidant balance [42, 43]. Despite extensive research over the years, the relationship between free radical generation, antioxidant enzymes and exercise in skeletal muscle remains controversial [44, 45]. These discrepancies may be related to differences in exercise mode, intensity, duration of the training program, and muscle fibre type. Skeletal muscles are highly heterogeneous. Each muscle fibre type has distinct metabolic characteristics and oxidative potential as well as antioxidant defence capacity [41]. In our study, as a result of fatigue stimulation in working muscle, there was a significant increase in the secondary products of lipid peroxidation and H_2O_2 compared to the intact (unstimulated muscle) muscle (Fig. 5). During intense (physical activity) contraction, the flow of oxygen through muscle cells is greatly increased. High levels of oxygen uptake (up to 100-fold) can lead to excessive ROS generation and are implicated in fatigue, muscle soreness, and myofibril disruption [45]. Moreover, another potential mechanism involved in the oxidative stress response to high-intensity exercise is the redistribution of blood flow, such as elevated blood flow in the heart, lung, and red slow-twitch muscle fibres, leading to increased mitochondrial respiration, which results in an increase in the production of ROS. We found that long-term electrical stimulation of the muscle induced a significant increase in *TBARS* and H_2O_2 content that led to an increase of *CAT* activity and *GSH* content in both fast- and slow-twitch muscle fibres. In this case, after C_{60}FAS administration, the oxygen metabolite concentration was significantly lower. This confirms the previous data regarding the protective effect of C_{60}FAS on the immune and antioxidant systems of the body in various pathologies [15, 46]. The mechanisms of effects of this drug can positively influence the processes of endurance and recovery of the active muscles, inactivating the products of its metabolism.

Increased amounts of *GSH* in the stimulated muscle (without drug administration and after its application) are evidence of the compensatory activation of the

endogenous antioxidant systems on the irritant action of sufficient strength (Fig. 5). Many studies showed that during intense stress, there is a significant decrease of reduced *GSH* and an increased concentration of its oxidative form in the myocardium and *m. soleus* [47, 48]. Simultaneously, contradictory data were obtained in the experiments studying endurance [47, 49]. It was found that under physical activity, the amount of reduced *GSH* in the *m. gastrocnemius* and *DVL* increase. It is likely that in *m. soleus*, a muscle with a high content of myoglobin, all metabolic and biochemical processes occur under aerobic conditions, which use a large number of mitochondrial enzymes, and the accumulation of oxidized *GSSG* does not have time to reduce [50]. At the same time, the above mentioned processes in the *m. gastrocnemius* occur anaerobically, in contrast to the *m. soleus*. This causes a slow oxidation process and increases the amount of reduced *GSH* [51, 52]. Under fatigue, after C_{60}FAS administration, the *GSH* content was somewhat reduced compared to the "fatigue" state, indicating a reduction in oxidative stress and a normalization of the pro- and antioxidant balance in rat muscle tissue (Fig. 5).

An increase of H_2O_2 during exertion leads to an increase in *CAT* enzyme activity that has a protective antioxidant function by catalysing the decomposition of hydrogen peroxide to water and oxygen. These results are confirmed by previously obtained data from acute experiments on rats with *DVL* stimulation [47, 52]. An increase of the enzyme activity in response to exercise was also shown in humans [53]. Moreover, some studies indicate an absence of any changes in *CAT* concentration in the muscles during physical activity [44, 54, 55]. In fact, several reports demonstrated decreases in catalase activity in both oxidative and mixed fibre limb muscles [56, 57]. In our study, after C_{60}FAS administration under fatigue development, the *CAT* activity was significantly reduced compared to pure fatigue and remained at the control level. It is hypothesized that C_{60}FAS influence the content and activity of endogenous antioxidants and prevent the occurrence of fatigue in actively contracting muscle, thereby contributing to maintenance of its normal physiological state.

Free radical processes increasing is the main pathogenic factor during skeletal muscles fatigue development [58]. Under significant physical activity there is highly overproduction of free radicals in muscle tissue that intensifies the processes of lipid peroxidation, cell membranes damage and antioxidant enzymes inactivation [59]. The active oxygen metabolites cause direct inhibition of respiratory chain mitochondrial enzymes and reducing the balance of ATP/ADF [59]. The above processes in the background of the lactate accumulation with subsequent development of acidosis and blockage of membrane Ca^{2+} channels lead to a pronounced energy deficit and a significant functional activity reduction of muscle tissue [60].

It is known that application of different nature exogenous antioxidants leads to a significant reduction of fatigue skeletal muscle during intense physical activity and increases the onset time of muscle fatigue under prolonged intense endurance exercise [10, 61, 62]. These data demonstrate the feasibility of using antioxidants to correct the level of oxidative stress in the muscle tissue under extreme influences on the body and its efficiency increasing. Since pristine C_{60} fullerenes, as previously shown in various models in vitro and in vivo [13, 15, 63], actively bind free radicals and display a powerful antioxidant properties of direct action, we can assume that the application of water-soluble C_{60} fullerenes led to the prooxidant-antioxidant balance normalization in the muscle tissue of rats and helped improve the dynamic parameters of muscle contraction.

Conclusion

The use of C_{60}FAS, even at a low therapeutic dose (0.1–0.15 mg/kg) leads to a reduction in the recovery time of the muscle contraction force (after its complete exhaustion state) on the one hand, and an increase in the time of the muscle active work (endurance) until fatigue development on the other. This result illustrates the effect of C_{60}FAS, along with other possible mechanisms, on prooxidant-antioxidant homeostasis in the muscle tissue of rats.

Abbreviations

C_{60}FAS: pristine C_{60} fullerene aqueous colloid solution; H_2O_2: hydrogen peroxide; *TBARS*: thiobarbituric acid reactive substances; *GSH*: reduced glutathione; *CAT*: catalase; ROS: reactive oxygen species; NAD^+: nicotinamide adenine dinucleotide; HCl: hydrochloric acid; AFM: atomic force microscopy; SANS: small-angle neutron scattering; FOX: ferrous ion oxidation xylenol orange; H_2SO_4: sulphuric acid; EDTA: ethylenediaminetetraacetic acid; DAC: digital to analogue converter; ADC: analogue to digital converter; ANOVA: analysis of variance; DVL: deep portion of vastus lateralis muscle; GSSG: glutathione disulfide.

Authors' contributions

IVV, AVM and NVB designed and performed the experiments, and the in vitro assays were performed by OOG. UR, PS and OAK were responsible for C_{60}FAS synthesis and characterization. TT helped with preparation of the manuscript and provided funding support. DMN and IVM helped collect and analyze data. YulP and AIK provided supervision and guidance throughout this work. The manuscript was written through contributions of all authors. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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The use of the animals was approved by the Ethics Committee of the Institute and performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

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C_{60} Fullerenes Diminish Muscle Fatigue in Rats Comparable to *N*-acetylcysteine or β-Alanine

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The aim of this study is to detect the effects of C_{60} fullerenes, which possess pronounced antioxidant properties, in comparison with the actions of the known exogenous antioxidants *N*-acetylcysteine (NAC) and β-Alanine in terms of exercise tolerance and contractile property changes of the *m. triceps surae* (*TS*) during development of the muscle fatigue in rats. The electrical stimulation of the *TS* muscle during four 30 min series in control rats led to total reduction of the muscle contraction force. Furthermore, the effects of prior intraperitoneal (i.p.) or oral C_{60}FAS application and preliminary i.p. injection of NAC or β-Alanine on muscle contraction force under fatigue development conditions is studied. In contrast to control rats, animals with C_{60}FAS, NAC, or β-Alanine administration could maintain a constant level of muscle effort over five stimulation series. The accumulation of secondary products and changes in antioxidant levels in the muscle tissues were also determined after the fatigue tests. The increased levels of lactic acid, thiobarbituric acid reactive substances and H_2O_2 after stimulation were statistically significant with respect to intact muscles. In the working muscle, there was a significant ($p < 0.05$) increase in the activity of endogenous antioxidants: reduced glutathione, catalase, glutathione peroxidase, and superoxide dismutase. Treated animal groups showed a decrease in endogenous antioxidant activity relative to the fatigue-induced animals ($P < 0.05$). Oral C_{60}FAS administration clearly demonstrated an action on skeletal muscle fatigue development similar to the effects of i.p. injections of the exogenous antioxidants NAC or β-Alanine. This creates opportunities to oral use of C_{60}FAS as a potential therapeutic agent. Due to the membranotropic activity of C_{60} fullerenes, non-toxic C_{60}FAS has a more pronounced effect on the prooxidant-antioxidant homeostasis of muscle tissues in rats.

Keywords: C_{60} fullerene, skeletal muscles fatigue, electrical stimulation, oxidative stress markers, antioxidant system

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Abbreviations: C_{60}FAS, pristine C_{60} fullerene aqueous colloid solution; CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; NAC, antioxidants *N*-acetylcysteine; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TS, *m. triceps surae*.

INTRODUCTION

Fullerenes are a new kind of organic compounds, consisting of carbon atoms with very attractive photo-, electrochemical and physical properties that can be used in many biological fields, which make fullerenes potential therapeutic agents for a wide range of applications in nanomedicine (Anilkumar et al., 2011; Dellinger et al., 2013; Castro et al., 2017). With respect to its electron donor and acceptor capability fullerenes can be effective antioxidants and radical scavengers (Bosi et al., 2003; Bakry et al., 2007). It was shown that under the influence of light, fullerene acts as a prooxidant (Kamat et al., 2000), generating singlet oxygen (Bosi et al., 2003), which can be used in photodynamic therapy of cancer and other diseases. Some fullerene derivatives exhibit inhibitory activity against human immunodeficiency virus reverse transcriptase and hepatitis C virus RNA polymerase (Mashino et al., 2005; Nakamura and Mashino, 2012), and can stabilize immune effector cells to prevent or inhibit the release of proinflammatory mediators, making them potential candidates for a variety of diseases, such as asthma, arthritis, and multiple sclerosis (Dellinger et al., 2013). Thus, fullerenes and their derivatives are excellent candidates for multiple functionalization.

It is known, that muscle fatigue is accompanied by ionic changes in action potentials (Allen et al., 2008) along with various metabolic disturbances in skeletal muscles, when reactive oxygen species (ROS) are formed (Halliwell and Gutteridge, 1989), excess lactic acid (LA) (Sahlin et al., 1987), and lipid peroxidation (Venditti and Di Meo, 1997). With excessive accumulation of these substances, oxidative stress occurs, which leads to significant functional disorders since various components of cells may be damaged. The damage could include changes in protein structures, nitrogenous bases, and destruction of membranes (Powers and Jackson, 2008). Muscle cells contain endogenous cellular defense mechanisms in the form of enzymatic and non-enzymatic antioxidants that can partially eliminate ROS (Sen et al., 1994; Banerjee et al., 2003). A changes in the level of enzymes (*SOD GPx*, and *CAT*) under loading was observed in both animals and humans (Sen, 1995). During exhausted loads, the amount of *SOD* increased in skeletal muscle (Powers et al., 1999), whereas the level of *GPX* activity had not changed (Brady et al., 1979) or was increased (Powers et al., 1999). Similar phenomena were observed concerning *CAT* activity when no changes were observed during exercise (Powers et al., 1994a), and there was an increase in activity under certain loads (Ji and Fu, 1992). Thus, the understanding of the mechanisms involved in the process of increasing antioxidant enzymes during exhaustive exercise has yet to be under debate.

Intense muscle loading contributes to rise of oxidative stress in muscle tissue (Powers et al., 1999). The presence of ROS inhibits the tricarboxylic acids cycle and disrupts the mitochondria electronic transport chain (Janero and Hreniuk, 1996). With intense physical activity, the rate of hydrolysis of ATP may exceed the rate of its resynthesis, which leads to a decrease in ATP and the formation of muscle fatigue effects (Graham et al., 1978; Sahlin, 1985). However, the synthesis of ATP during training can be supported by antioxidants supplementation, which accelerate the process of muscle recovery after fatigue. There are evidences demonstrated a moderately beneficial effect of NAC (Supinski et al., 1997; Sandstrom et al., 2006) and β-Alanine (Stout et al., 2007; Ghiasvand et al., 2012; Summermatter and Handschin, 2012; Schnuck et al., 2016) supplementation for exercise with a substantial contribution from oxidative metabolism. It was shown unspecific antioxidant activity of NAC with increase GSH synthesis and reduce muscle-derived ROS levels during contraction (Sandstrom et al., 2006). Another study suggested a delay accumulation of lactate during exercise and increasing time to exhaustion under β-Alanine supplementation (Summermatter and Handschin, 2012). In our previous study, we showed a facilitation effect of water-soluble C_{60} fullerenes on the removal of some symptoms of skeletal muscle fatigue (Prylutskyy et al., 2017). So, we consider that because of novel field of C_{60} fullerenes application (muscle fatigue) it was important to assessed the adequacy and efficiency of C_{60} fullerene protective properties against oxidative damage in comparison with the action of known antioxidant NAC and β-Alanine. It was hypothesized that due to its unique chemical structure and bioactivity a water-soluble C_{60} fullerenes would have predominant influence on prooxidant-antioxidant homeostasis of rat muscle tissue. In our the previous work we detected the effect of the C_{60} fullerenes in the short-term period after intramuscular injection (Prylutskyy et al., 2017), in this study we determined the most optimal way of its application and investigated the effect of C_{60} fullerenes after preliminary administration, which implies its preventive use in case of muscular fatigue.

MATERIALS AND METHODS

Material Preparation and Characterization

A highly stable C_{60}FAS at a maximum concentration of 0.15 mg/ml was prepared as described earlier (Scharff et al., 2004; Ritter et al., 2015). Briefly, for the preparation of C_{60}FAS we used a saturated solution of pristine C_{60} fullerene (purity >99.99%) in toluene with a C_{60} 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 ultrasonic bath. The procedure was continued until the toluene had completely evaporated and the water phase became yellow colored. Filtration of the aqueous solution allowed to separate the product from undissolved C_{60} fullerenes.

DLS Measurements

Measurements of the hydrodynamic size distribution for C_{60} fullerenes in aqueous solution were performed by dynamic light scattering (DLS) on a Zetasizer Nano-ZS90 (Malvern, Worcestershire, United Kingdom) at room temperature. A DLS instrument equipped with a HeNe laser (max 5 mW) operating at a wavelength of 633 nm was used. The measurements were performed at a 90° scattering angle. The autocorrelation function of the scattered light intensity was analyzed with Static Light Scattering software.

Zeta Potential Measurements

Zeta potential measurements for C_{60}FAS were carried out on a Zetasizer Nano-ZS90 (Malvern, Worcestershire, United Kingdom) at room temperature. The results were evaluated using the Smoluchowski approximation, which is known to be valid only for spherical-like particles.

Procedure and Experimental Groups

Experiments were performed on male Wistar rats weighing 280–350 g with ages ranging from 4 to 6 months. The animals were purchased from a state-controlled animal farm through the common animal facility of Bogomoletz Institute of Physiology (Kyiv). The experimental animals were housed in Plexiglas cages (four rats per cage) and kept in an air-filtered and temperature-controlled (20–22°C) room. The rats received a standard pellet diet and water *ad libitum*. The present study was approved by the Ethics Committee of the Institute and performed according to the European Communities Council Directive of November 24, 1986 (86/609/EEC).

All animals were randomly divided into 7 groups: 1st – fatigue-induced animals (n = 6); 2nd – vehicle-injected [fatigue-induced rats with a preliminary intraperitoneal (i.p.) injection of 0.3 ml of saline solution, n = 6]; 3rd – C_{60}FAS-injected [F-injection; fatigue-induced rats with a preliminary i.p. injection of 0.3 ml (0.14 mg/kg) of C_{60}FAS, n = 6]; 4th – animals with oral administration of C_{60}FAS [F-drinking; fatigue-induced rats with a preliminary (within 5 days, 0.225 mg/kg per day) oral introduction of C_{60}FAS, n = 6]; 5th – NAC-injected (fatigue-induced rats with a preliminary i.p. injection of 150 mg/kg of NAC dissolved in saline solution, n = 6); 6th – β-Alanine-injected (β-Al-injection; fatigue-induced rats with a preliminary i.p. injection of 110 mg/kg of β-Alanine dissolved in saline solution, n = 6); and 7th – intact animals (rats were used only for biochemical studies, n = 6).

It should be noted that the toxicity of the pristine C_{60} fullerenes is strictly dependent on their size (the degree of aggregation in the water). In our previous works (Prylutska et al., 2009, 2017; Tolkachov et al., 2016), we investigated in detail the *in vitro* toxicity of C60FAS. We can conclude that at a maximum concentration of 0.15 mg/ml, C60FAS (the diameter of nanoparticles is up to 50 nm) does not exhibit any genotoxicity and cytotoxicity to various types of cells, including human ones. It is important to note that the doses of C_{60}FAS used in this study do not present any acute or subacute toxicity in animals: they were significantly lower than the maximum tolerated dose of pristine C_{60} fullerene, which was found to be 5 g/kg both for oral or i.p. administration to rats (Gharbi et al., 2005). Toxic or lethal effects were not observed in the studies of the impact of C_{60} fullerenes after oral administration to rats at a total dose of 2 g/kg for 14 days (Mori et al., 2006). Moreover, according to our previous data (Tolkachov et al., 2016) C_{60}FAS at the concentrations up to 24 μg/ml does not manifest any *in vitro* toxic effect on human mesenchymal stem cells. C_{60}FAS (0.1 mg/ml) does not induce DNA strand breaks in the human lymphocytes as revealed by the comet assay (Prylutska et al., 2017). Finally, the authors (Świdwińska-Gajewska and Czerczak, 2016) state that oral exposure of pristine C_{60} fullerene nanoparticles does not lead to major adverse effects because they were not mutagenic and genotoxic in experimental research. The pristine C_{60} fullerene is characterized by low toxicity and it does not pose a risk in the occupational environment.

Fatigue of the *TS* of rat was induced by electrical stimulation of *n. tibialis*. C_{60}FAS, NAC, β-Alanine or saline solution were administered 1 h prior to electrical stimulation. After the experiment, the *TS* of all animals in all groups were removed for biochemical analysis.

The animals were anesthetized with ketamine (100 mg/kg "Pfizer", United States) combined with xylazine (10 mg/kg, "Interchemie," Holland), tracheostomized and artificially ventilated (out of necessity). The left and right *TS* muscles were separated from the surrounding tissue, their tendons were detached at the distal insertions, and a small bone chip from the heel was left behind. The *n. tibialis* was separated from the tissue and cut proximally, and all branches of the nerve, except nerves innervating the *TS*, were cut. This nerve was mounted on a bipolar platinum wire electrode for electrical stimulation. The hindlimb muscles and nerves were covered with paraffin oil in a pool formed by the skin flaps. The ECG and heart rate were continuously monitored. Pools with mineral oil were maintained at 37–38°C using radiant heat. The *TS* muscle was connected via the Achilles tendon to the servo-control muscle puller. A linear motor under servo-control was used as the muscle puller. The muscle tension was measured by semi-conductor strain gauge resistors glued on a stiff steel beam mounted on the moving part of the linear motor. The stiffness of the puller exceeded 0.06 N/mm, whereas the time constants of the length transients did not exceed 60 ms.

To induce muscle fatigue, a series (30 min duration) of intermittent high-frequency electrical stimulations was used (**Figures 1A–D**), separated by rest intervals of 15 min. Four stimulation series were used for the rats in group 1, and 5 stimulation series were used for the other groups of animals. Each series consisted of trains of 0.2 ms rectangular pulses at a rate of 40 s^{-1} at a 12.4 s duration, and the series were separated by 5 s intervals of rest (**Figure 1D**, in a circle). The stimulus current was set to 1.3–1.4 times higher than the motor threshold. At the end of the 12.4 s stimulation, the muscle was stretched, and the changes in length had a bell-shaped form (one period of 4 Hz sinusoidal signal with corresponding phase locking) with a 3.5 mm amplitude and 2 s duration (**Figure 1D**, bottom row in a circle). The muscle reaction to the stretching appeared to be a tension increase after continuous stimulation. These stretches were applied before the post-stimulation twitches to remove, or at least diminish, the after-effects remaining from continuous stimulation (Kostyukov et al., 2000). The command signal to the muscle puller was derived from a DAC and was adjusted by a scaling amplifier and low-pass filter (0–100 Hz). In parallel, two analog signals (muscle tension and length) and pulse signals (stimulation pulses) were sampled via corresponding ADC channels. The signals were collected by PC using an input-output interface device (CED Power 1401) with 12-bit resolution.

Data acquisition was performed using the program "Spike2" (CED). Input signals were digitized at rates of 5 kHz (muscle

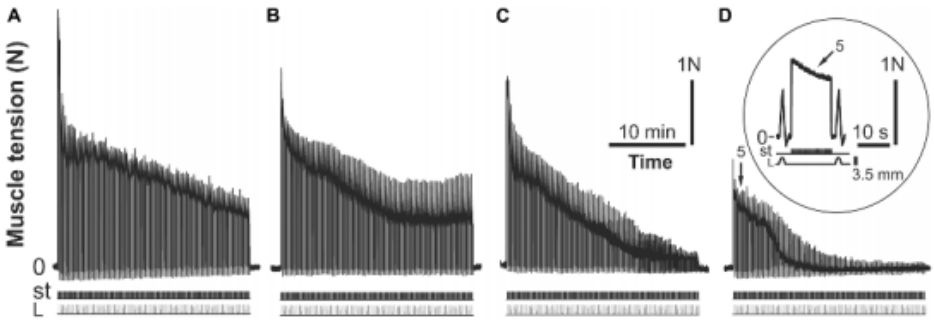

FIGURE 1 | Strength of the *m. triceps surae* (*TS*) contractions during consecutive (I–IV) series of 30-min intermittent stimulations of the *n. tibialis*; the rat belongs to the fatigue-induced group of animals (group 1) **(A–D)**. The 5th individual tetanic contraction of *TS* in the IV stimulation series is presented in a circle at a higher time scale on **(D)**. P – muscle force (Newton, N), st – stimulation mark, L – muscle length (stretching, mm).

tension) and 1 kHz (other signals). Data analysis, including statistical treatment and graph plotting, was performed using the program Origin 8.0 (OriginLab Corp., United States).

Biochemical Experiment

After acute exercise, the excised muscles (soleus and gastrocnemius) were rapidly dissected, free of fat and tendons were removed, and the muscles were divided into several portions and stored in liquid N_2. For *GSH* (reduced glutathione) analysis, the tissue samples were transferred to a medium containing 1N perchloric acid (1:10 w/v) and homogenized with a motor-driven Potter-Elvehjem glass homogenizer. Resultant homogenate was centrifuged at 10,000 *g* for 10 min (4°C). *GSH* content was measured spectrophotometrically (Sedlak and Lindsay, 1968). For the activities of enzymes, H_2O_2 and lipid peroxidation assays, the muscle samples were thawed and homogenized in 50 mM phosphate buffer with 2 mM *EDTA* (pH 7.4) at 4°C (1:9 w/v). The homogenates were then centrifuged then for 15 min at 15,000 *g* (4°C), and the post-mitochondrial supernatant was stored at −70°C.

Oxidative damage in tissue was measured using a *TBARS* assay. *TBARS* were isolated by boiling tissue homogenates for 15 min at 100°C with thiobarbituric acid reagent (0.5% 2-thiobarbituric acid/10% trichloroacetic acid/0.63 M/dm^3 hydrochloric acid) and measuring the absorbance at 532 nm. The results were expressed as nM *TBARS*/mg protein using $\varepsilon = 1.56 \times 10^5$ dm^3/M/cm (Buege and Aust, 1978). The data on ROS formation were obtained from dichlorofluorescein (DCF) fluorescence. The tissue homogenates were loaded for 20 min at 37°C with non-fluorescent probe (2′,7′-dichlorodihydrofluorescein diacetate, DCFHDA) which is known to be decomposed in cells to give dichlorofluorescein upon oxidation by ROS, primarily hydroperoxide and superoxide anion. The final concentration of DCFH-DA was 10 μM. DCF formation was followed at the excitation wavelength of 488 nm and emission wavelength of 525 nm for 30 min by using a Hitachi F-2000 fluorescence spectrometer. The rate of DCFH-DA conversion to DCF was linear for at least 60 min, corrected with the autoxidation rate of DCFH-DA without protein. All assays were carried out in duplicates. Fluorescence was expressed as arbitrary fluorescence units.

H_2O_2 concentration in the tissue homogenates was measured using the FOX method, which is based on the peroxide-mediated oxidation of Fe^{2+}, followed by a reaction of Fe^{3+} with xylenol orange (o-cresolsulfonephthalein 3′,3″bis[methylimino] diacetic acid, sodium salt). This method is extremely sensitive and used to measure low levels of water-soluble hydroperoxide present in the aqueous phase. To determine the H_2O_2 concentration, 500 μL of the incubation medium was added to 500 μL of assay reagent (500 μm ammonium ferrous sulfate, 50 mm H_2SO_4, 200 μm xylenol orange, and 200 mm sorbitol). The absorbance of the Fe^{3+}-xylenol orange complex (A_{560}) was detected after 45 min. Standard curves of H_2O_2 were obtained for each independent experiment by adding variable amounts of H_2O_2 to 500 μL of basal medium mixed with 500 μL of assay reagent. The data were normalized and expressed as μm H_2O_2 per mg protein (Wolff, 1994).

Catalase activity was measured by decomposition of H_2O_2, which was determined by a decrease in absorbance at 240 nm (Aebi, 1983).

Reduced glutathione was determined using Ellman's reagent. One milliliter of supernatant was treated with 0.5 ml of Ellman's reagent (5.5′-dithio-bis-nitrobenzoic acid in abs. ethanol) and of 0.4 M *Tris HCl* buffer with 2 mM *EDTA*, pH 8.9. The absorbance was read at 412 nm in a spectrophotometer (Sedlak and Lindsay, 1968).

Manganese-superoxide dismutase (*Mn-SOD*) activity was estimated by the method of Misra and Fridovich (1972), which is based on the inhibition of autoxidation of adrenaline to adrenochrome by the *SOD* contained in the examined samples. The mitochondrial samples were preincubated at 0°C for 60 min with 6 mM *KCN*, which produces total inhibition of *Cu, Zn-SOD* activity. The results were expressed as the specific activity of the enzyme in units per mg protein. One unit of *SOD* activity was defined as the amount of protein causing 50% inhibition of the conversion rate of adrenaline to adrenochrome under specified conditions.

The activity of selenium-dependent *GPx* was determined according to the method of Flohé and Günzler (1984). Briefly, the reaction mixtures consisted of 50 mM KPO_4 (pH 7.0), 1 mM *EDTA*, 1 mM NaN_3, 0.2 mM *NADPH*, 1 mM *GSH*, 0.25 mM H_2O_2, and 226 U/ml glutathione reductase, and the rates of *NADPH* oxidation followed at 340 nm.

Lactic acid was determined in muscle tissue after deproteinization with 6% (wt/wt) perchloric acid by sitting the tissue on ice for 15 min and then centrifuging the tissue at 14,000 *g* for 5 min. The supernatant was neutralized with 5 M K_2CO_3, clarified again to remove potassium perchlorate, and stored at −70°C. The assay mixtures contained glycine/*EDTA*/hydrazine hydrate buffer (pH 9.5), 0.05 mM *NAD*, 10 units of lactate dehydrogenase, and sample (100 µl), and the mixtures were incubated at 37°C for 20 min. The *LA* concentration was determined spectrophotometrically at 340 nm in a 1.0-ml total reaction volume (Hohorst, 1970).

Protein concentration was estimated with the Bradford method using bovine serum albumin as a standard. All chemicals were purchased from Sigma, Fluka and Merck and had the highest available purity.

Statistical Analysis

In the electrophysiological study, each stimulation series was averaged (100 stimulations in one series). The average value of the first series was set to 100%, and the other series were normalized in relation to this and presented graphically for one of hindlimb. During experiments, it was found that till the moment of a drastic decrease of TS contraction force control animals maintain a certain force level of muscular contraction only during three series of electrical stimulation. To confirm the muscle fatigue factor, data from four stimulation series were taken for the study. Some animals from groups 3–6 to maintain a certain force level of muscular contraction until 5–6 series of electrical stimulation. Therefore, for further analysis, data from the first 5 series were taken.

Mean values (mean ± SD) of the *TS* muscle strength after C_{60}FAS, NAC, β-Alanine, saline solution induction or without any induction were compared using two-way statistical analysis of variance (ANOVA). The factors of variation included two conditions: time and the effects of the C_{60}FAS (NAC, β-Alanine or non-injected). A Bonferroni *post hoc* analysis was used to determine the differences between groups. The level of significance was set at $P < 0.05$.

Biochemical data are expressed as the means ± SEM for each group. The differences among experimental groups were detected by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Values of $P < 0.05$ were considered significant.

RESULTS

It has been established that the size of water-soluble C_{60} fullerene particles directly correlates with their cytotoxicity and biological properties (Lyon et al., 2006; Prylutska et al., 2009; Song et al., 2011; Lalwani and Sitharaman, 2013; Zhang et al., 2015). Depending on the size, water-soluble C_{60} fullerene particles can penetrate through the plasma membrane into the cell or be adsorbed on the surface of the membrane (Foley et al., 2002; Schuetze et al., 2011; Franskevych et al., 2017). In this regard, the main advantage of using pristine C_{60} fullerenes as powerful antioxidants (Gharbi et al., 2005; Prylutska et al., 2008) is their ability to be localized preferentially to mitochondria, which generates a substantial amount of cellular ROS (Foley et al., 2002; Youle and Karbowski, 2005). Thus, since the size of C_{60} fullerene particles and stability of their aqueous solution (the degree of aggregation in water) may influence their bioactivity, the DLS and zeta potential studies of C_{60}FAS were performed.

DLS and Zeta Potential Studies

The DLS results for investigated C_{60}FAS clearly demonstrate that there was a monomodal nanoparticle size distribution in a (15–30) nm range, i.e., these nanoparticles in particular have specific bioactivity. This result is similar to our previous probe microscopic data, which directly correlate with C_{60} fullerene bioactivities (Prylutska et al., 2014; Skamrova et al., 2014).

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 dispersal system. The value of zeta potential for C_{60}FAS was equal to −23 mV, which agrees well with our previously published data (Ritter et al., 2015). A high negative charge for colloid nanoclusters (or, more strictly, the electrostatic repulsion between the negatively charged nanoclusters) seems to play a significant role in the stabilization of C_{60}FAS (i.e., it disfavors the aggregation and makes the solution electrically stable).

Electrophysiological Experiments

As a result of intermittent high-frequency electrical stimulation (30 min duration, 40 Hz) of *TS* muscle in the rats of the first and second groups during four series, the reduction of the force contraction was recorded until the muscle ceased to demonstrate clear contractions. **Figure 1** shows an example of the *TS* force response changes in one animal of the first group during fatigue development. In this case, the dynamics of changes in muscle force reflected fatigue development and a drop in amplitude in single contractions. In the first series of stimulations, a gradual reduction of *TS* activity level was observed over 30 min. In the second series of stimulations, which were started after a 15 min rest, the amplitudes of tetanic contractions were somewhat recovered; however, they did not reach the initial level, continuing to decrease. Within the third series of stimulations (after 90 min of electrical stimulation), an abrupt drop in the muscle force was observed. Within this time interval, the maximum significant strength level decrease ($P < 0.05$) was observed with respect to the first series of stimulations (**Figure 2**). After another interruption between stimulations, the isometric muscle force contraction continued to decline without further recovery. At the same time, such a decrease in muscle force response was statistically significant ($P < 0.05$) within the IV series of stimulations with respect to that one in the I, II, and III series (**Figure 2**). Note that statistically significant differences

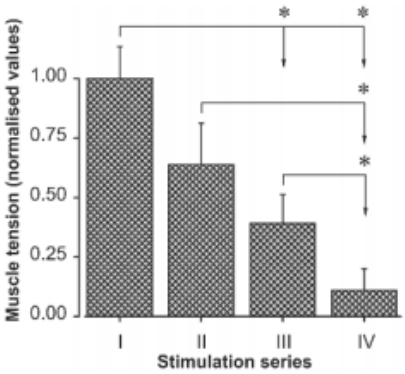

FIGURE 2 | Changes in the *TS* muscle strength in the I–IV series of fatigue stimulation. There are normalized averaged force (mean ± SD) (normalization with respect to the first series) values. The averaging procedure has been applied to the data registered in six animals of the fatigue-induced group of rats (group 1), and the data were averaged with respect to the corresponding stimulation series. Asterisks denote significant differences ($P < 0.05$) between the muscle strength of different electrical stimulation series.

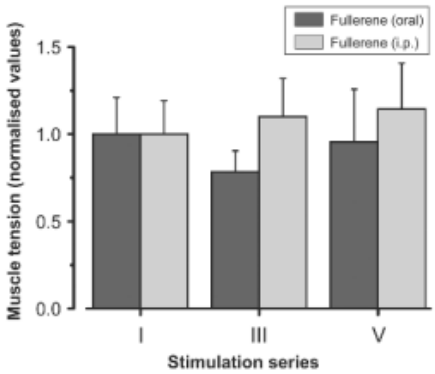

FIGURE 3 | Averaged characteristics (mean ± SD) of the normalized (with respect to the first stimulation series) values of the muscle strength after preliminary intraperitoneal injection and oral administration of C_{60}FAS (*dark gray* and *light gray bars*, respectively). I, III, and V – series of electrical fatiguing stimulation. Values of six animals in each group were averaged according to stimulation series. Note the absence of significant differences between data obtained in rats of groups 3 and 4.

($P > 0.05$) in the strength of muscle contractions between rats in groups 1 and 2 were not found.

For assessments of the effectiveness of administration method of the C_{60}FAS under conditions of fatigue stimulation it was made a comparison of the changes in the force levels developed by muscles in animals with C_{60} fullerene i.p. injection (F-injection) and in animals that drank C_{60} fullerene for 5 days (F-drinking) (**Figure 3**). The analysis did not reveal any significant differences in the muscle strength after studying the effects of these substance administration methods on muscle fatigue (F-injection vs. F-drinking). In this case, under conditions of both F-injection and F-drinking, the muscle maintained the same constant force level during the first series of fatigue stimulation (30 min). In animals with a C_{60} fullerene i.p. injection during the third series of stimulations, the force level was slightly higher compared to the group of animals that drank C_{60}FAS. Under conditions of further fatigue stimulation (V series) with F-injection and F-drinking, the difference in the level of the developed effort decreased (**Figure 3**). Thus, the absence of statistically significant differences ($P > 0.05$) made it possible for us to conduct further analyses of fatigue development using only F-drinking administration, since it is non-invasive and potentially more practical for future application.

Further analysis suggested the force fatigue contractions on the background of a separate action of C_{60}FAS attach be compared to exogenous antioxidants NAC and β-Alanine (**Figure 4**). The data obtained in these experiments (**Figures 4B,F**) indicate that a reduction in the developed force in the animals of the F-drinking, the NAC-injected and the β-Al-injected group was slower compared to fatigue-induced or vehicle-injected animals. Significant differences ($P < 0.05$) in muscle strength changes between control animals (groups 1 and 2) and experimental rats (groups 4–6) appeared after the second stimulation series. In groups 4–6, the muscle maintained a constant level of developed effort through the whole fatigue stimulation, which was demonstrated using native records of its force characteristics (**Figures 4A,C,E**). The animals of the F-drinking group held a stationary level of force longer than the NAC-injected and β-Al-injected animals. The dynamics of the force changes was similar in almost all animals of this group (**Figure 4B**). In the NAC-injected animals during the III series, there was decrease in the muscle strength amplitude along with further stabilization, but these effects were not significant (**Figure 4C**). It should be noted that in two animals of this experimental group, recovery of the muscle contraction force level to the force level at the initial fatigue stimulation stages occurred (**Figures 4C,D**). At the same time, in the F-drink group during the V series of fatigue stimulation, muscle strength recovery was also observed, and in some animals, its increase was recorded (**Figure 4B**). In the β-Al-injected group, the developed force was maintained at a steady-state level within each series of stimulations (**Figure 4E**). At the end of the I series of stimulation, there was a slight decrease in muscle force with some recovery in the III series of stimulations. However, during further stimulation (V series), the level of the muscle force was slightly lower compared to previous series (**Figure 4E**). In this case, in all animals of this experimental group a similar change in muscle force was observed (**Figure 4F**).

To compare the action of the C_{60}FAS and antioxidants on the force of contractions during fatigue development, a statistical analysis was performed (**Figure 5**). In NAC-injected animals, during the initial series of stimulation, there was maintenance of the force at a constant level followed by some decrease. The characteristics of fatiguing muscle contractions were somewhat different in the F-drinking group. In these experiments, the level of muscle force, after some initial reduction in the I series

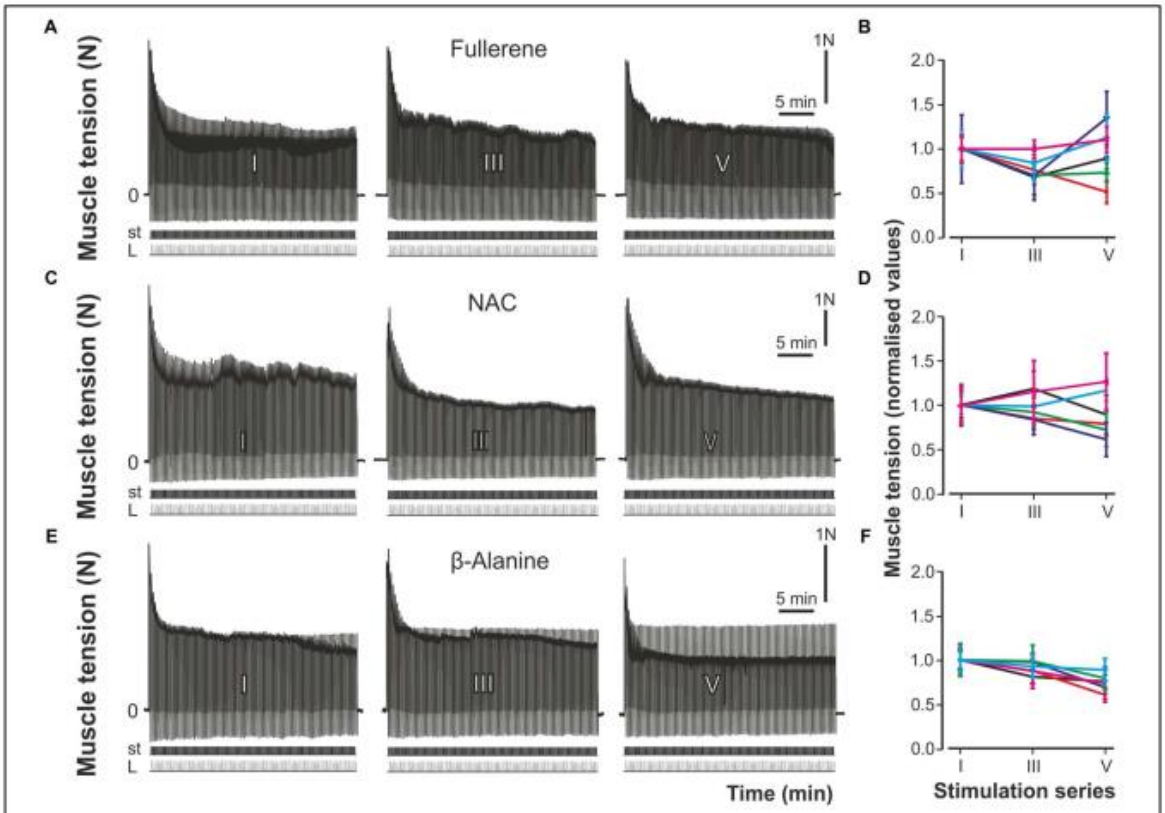

FIGURE 4 | Strength of the muscle contractions after preliminary oral administration of C_{60}FAS and injection of NAC and β-Alanine. **(A,C,E)** Protocol of registration of the muscle contractions after preliminary C_{60}FAS oral administration, NAC- and β-Al-injection, respectively; **(B,D,F)** – results of six fatigue tests in the groups of animals with preliminary C_{60}FAS oral administration, NAC- and β-Al-injection, respectively. I, III, and V – series of electrical fatigue stimulations, P – muscle force (Newton, N), st – stimulation mark, L – muscle length (stretching).

of stimulations, was further recovered and almost reached the control values. In the β-Al-injected group, a gradual decrease of the muscle force was observed. However, it should be noted that there was no significant difference in the muscle force for series conducted within the same animal group, as well as between groups (NAC-injected, F-drink, β-Al-injected) (**Figure 5**).

Biochemical Experiments

After the fatiguing tests, accumulation of secondary products and changes in antioxidant levels in the muscle tissues were determined (**Figure 6**). The obtained data clearly demonstrate the increased level of metabolic product (*LA*), markers of peroxidative and oxidative stress (*TBARS*, H_2O_2) and ROS formation after stimulation that indicates the occurrence of muscle fatigue (**Figure 6**). This increase was statistically significant with respect to the intact muscles ("*norm*") and consisted of 61% for ROS formation, 78% for *TBARS*, 115% for H_2O_2, and 198% ($P < 0.05$) for *LA*. In turn, in response to these changes in the working muscle, there was a significant ($P < 0.05$) increase in endogenous antioxidant

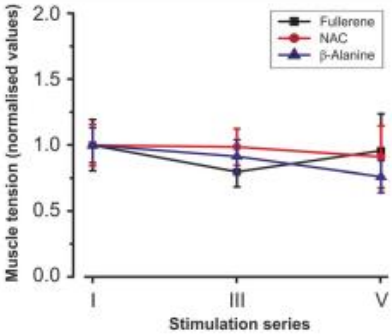

FIGURE 5 | Averaged characteristics (mean ± SD) of normalized (with respect to the average values in the first stimulation series) values of the muscle strength after preliminary C_{60}FAS oral administration, NAC- and β-Al-injection. Values of six animals in each group were averaged by stimulation series. Note the absence of significant differences between the data obtained in the rats of these groups. I, III, and V – a series of electrical fatigue stimulations.

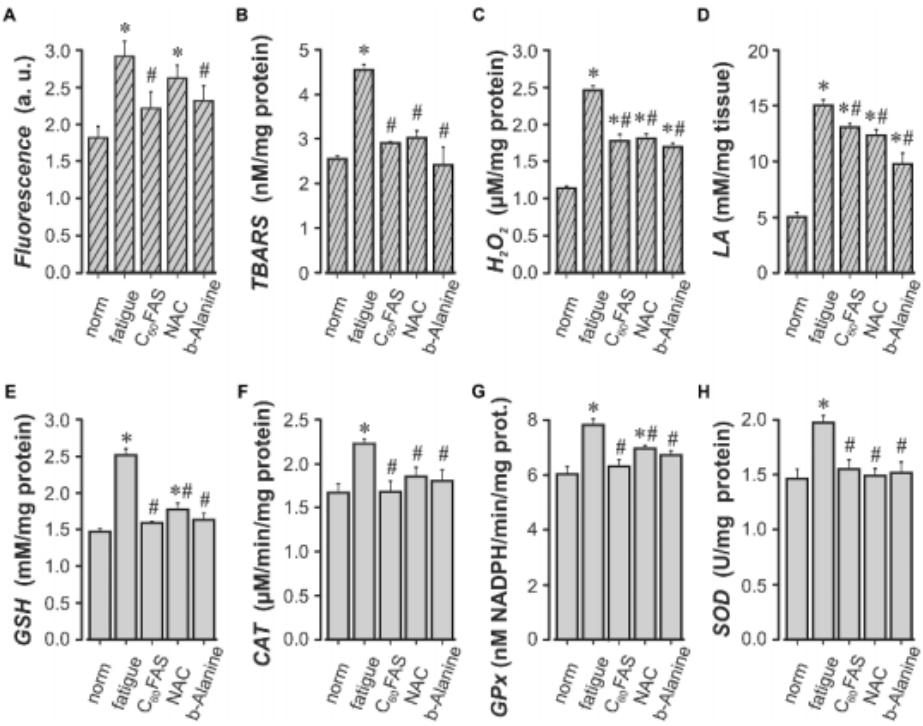

FIGURE 6 | Indicators of the prooxidant-antioxidant balance in the *m. triceps surae* (*TS*) of rats. ROS formation **(A)**, the concentration of thiobarbituric acid reactive substances (*TBARS*) **(B)**, hydrogen peroxide (H_2O_2) **(C)**, lactic acid (*LA*) **(D)**, reduced glutathione (*GSH*) **(E)**, catalase (*CAT*) **(F)**, glutathione peroxidase (*GPx*) **(G)** and superoxide dismutase (*SOD*) **(H)** are in intact animal muscles (norm), *TS* fatigue-induced animals (fatigue) and *TS* fatigue-induced animals after C_{60}FAS oral administration (C_{60}FAS), NAC-injection (NAC) and β-Al-injection (β-Alanine), respectively. Values are the means ± SEM, $n = 6$. *$P < 0.05$ vs. "norm"; #$P < 0.05$ vs. "fatigue."

activity, including *GSH* (71%), *CAT* (18%), *GPx* (28.5%), and *SOD* (34%).

In animals that drank C_{60}FAS, ROS formation and concentrations of *TBARS*, H_2O_2 and *LA* were significantly lower at 24.1, 36.1, 27.8, and 13.1% ($P < 0.05$), respectively, compared to the "*fatigue*" group. Similar changes in the level of these marker concentrations were also observed in the NAC-injection and β-Al-injection groups. A similar dynamic has been revealed in changes in the activity of antioxidant enzymes. Under conditions of fatigue development, the level of *GSH*, *CAT*, *GPx*, and *SOD* activity significantly increased by 71.4, 18.6, 28.5, and 34.2% relatively to the "*norm*," respectively. The animal groups F-drinking, NAC-injection and β-Al-injection showed a decrease in endogenous antioxidants activity relative to the "*fatigue*" group ($P < 0.05$). Furthermore, in rats that drank C_{60}FAS, the activity levels of *GSH*, *CAT*, and *GPx* were smaller.

DISCUSSION

In this study, we compared the effects of an aqueous colloid solution of pristine C_{60} fullerene with the action of the NAC and β-Alanine on exercise tolerance and contractile properties of rat *TS* during development muscle fatigue. This experimental approach allowed us to analyze and compare the characteristics of the force parameter changes of muscle contraction under conditions with the same fatigue stimulation pattern applied after oral introduction of C_{60}FAS or separate i.p. injections of C_{60}FAS, NAC and β-Alanine. The results showed a statistically significant reduction in muscle force in all animals without prior administration of C_{60}FAS, NAC, and β-Alanine (**Figure 2**). To maintain the normal functional and physiological state of the muscle during the dynamic work performance, the duration of its recovery and active rest are very important factors (Harris and Sale, 2012). An insignificant decrease in the muscle contraction force compared to the control occurred in animals that drank C_{60}FAS and in both NAC- or β-Al-injected rats (**Figure 5**). This outcome supported our previous finding that fullerene can lower the effects of fatigue development and promote force maintenance at a constant level (Prylutskyy et al., 2017). At the same time, in most animals of the F-drinking group, there was a recovery of the force level after some decrease, whereas in the NAC- and β-Al-injected groups, only an insignificant decline occurred (**Figures 4B,D,F**). A definite recovery of the contractile

ability was noticed in only two animals from the NAC-injected group (**Figure 4D**). These data on the effects of C_{60}FAS as a potent antioxidant under fatigue development conditions are in accordance with the data that were obtained earlier (Prylutskyy et al., 2017). In this case, one can speak about the predominant influence of C_{60} fullerene on muscle strength characteristics during fatigue development compared to the action of other investigated exogenous antioxidants, such as NAC or β-Alanine.

In our study, we showed that fatigue stimulation in the working muscle led to an increase in the metabolic products (*LA*) and the intensification of the oxidative processes, a namely a significant increase in ROS formation and lipid peroxidation, which occurs simultaneously with an increase in *CAT* and *GSH* activity in both fast and slow twitch muscles fibers relative to the intact muscle (**Figure 6**). The increased *LA* level further reduced the pH, which could induce various biochemical and physiological effects during muscular contractions, including glycolysis, phosphofructokinase, and calcium release (Wang et al., 2012). Therefore, *LA* is an important marker for evaluate the degree of fatigue of a living organism. In the group of animals that drank C_{60}FAS, attenuation of oxidative stress was observed (i.e., a decrease in ROS generation and TBARS concentration). This outcome was confirmed by the data obtained earlier on the effects of C_{60} fullerenes for the prooxidant–antioxidant homeostasis of rat muscle tissue (Prylutskyy et al., 2017). Similar changes in the number of metabolites and peroxide oxidation markers were observed in the NAC-injected and β-Alanine-injected groups. It has been supported that β-Alanine supplementation increases carnosine levels and decreases lactate responses after high-intensity exercise in rat muscles (Culbertson et al., 2010). In skeletal muscles, carnosine acts as a pH buffer and functions as an antioxidant (Boldyrev et al., 1993), which suggests that there is a potential role for carnosine for reversing or limiting the effects of oxidative stress and cellular senescence. Therefore, it cannot be excluded that the β-Alanine intake results in decrease of lactate and H^+-ion production. The skeletal muscles from NAC-injected rats showed a low content of *TBARS*, suggesting a reduced muscle fiber disruption due to cell membrane lipid peroxidation as compared with non-injected rats. This protective effect of NAC is due to direct scavenging of ROS and *GSH* synthesis enhancement (Aruoma et al., 1989; Supinski et al., 1997; Sen and Packer, 2000).

The important role of *GSH* in protecting against exercise-induced oxidative stress has been demonstrated in several studies (Sen et al., 1994; Leeuwenburgh et al., 1997). The increased amount of *GSH* in the stimulated muscle (without the C_{60}FAS, NAC, or β-Alanine administration and after their application) indicates compensatory activation of the endogenous *GSH* antioxidant system on the action of the stimulus (**Figure 6E**). In some studies, it has been shown that during intense loads, there is a significant decrease in *GSH* content in *m. soleus* and an increase in *GSH* content in *m. deep vastus lateralis* (*DVL*), but did not alter *GSH* status in the liver or plasma (Leeuwenburgh et al., 1997). Although other studies indicate an increase in the concentrations of *GSH* and *GSSG* in *m. soleus*, less in *DVL* and *m. superficial vastus lateralis* (*SVL*). However, the *GSSG*/*GSH* ratio does not change significantly because *GSSG* can be reduced to *GSH* by glutathione reductase. Furthermore, exercising skeletal muscles appear to increase GSH import from plasma (Ji et al., 1992) as well as the synthesis of *GSH* in the liver from endo- or exogenous amino acids, which adds most of the circulating *GSH* (Meister and Anderson, 1983), that ensures plasma GSH homeostasis despite enhanced tissue *GSH* use. Our experiments showed an increase in *GSH* activity during fatigue and a decrease in *GSH* under the actions of C_{60}FAS, NAC, or β-Alanine. This outcome is consistent with the data of other authors who have shown that the use of NAC, in particular, decreases exercise-induced *GSSG*, and blood lipid peroxidation in rats (Sen et al., 1994) improves muscle contractile functions as well as reduces low frequency fatigue in the diaphragm muscle (Shindoh et al., 1990) and human leg muscles (Reid et al., 1994).

An increase in the level of H_2O_2 activity during muscle contractions led to an increase in the *CAT* enzyme concentration (**Figure 6F**). These data also support studies performed earlier in rats (Ji and Fu, 1992; Hollander et al., 1999). It was shown that CAT activities were significantly elevated after exhaustive exercise with or without hydroperoxide injection in muscle and not in liver (Ji and Fu, 1992). Previously, it was reported that after the acute stage of the exercise, *CAT* was significantly higher in *m. soleus* than in *DVL* and *SVL*. Furthermore, the exercise at moderate intensities elicited significant increases in *CAT* activity in *DVL* (Ji et al., 1992). However, some studies have reported no changes in muscle *CAT* with training (Powers et al., 1994a; Radák et al., 1995), and a few studies even reported a decrease in *CAT* activities in *m. soleus* of the adult and old rats (Leeuwenburgh et al., 1994). In our study, during fatigue under NAC or β-Alanine treatment, the effects of *CAT* activity decreased with respect to "*fatigue*," but exceeded the control values. However, in animals that drank C_{60}FAS, *CAT* activity remained at the control level, which indicated that there was a greater compensatory effect of C_{60} fullerenes.

An increase in the concentration of oxidative process markers led to an increase in the activity of *GPX* in the working muscle (**Figure 6G**). However, with using of NAC and β-Alanine, the activity of this enzyme decreased relatively to "*fatigue*," and in the group of F-drinking, it was practically restored to normal. Data about *GPX* activity remain controversial. Powers et al. (1994a) showed an increase in *GPX* activity in red *m. gastrocnemius* after endurance training in rats, whereas *m. soleus* and white *m. gastrocnemius* revealed no training effect. The magnitude of the GPX increase was directly related to exercise duration but independent of intensity. Thus, the *GPX* activity has demonstrated variable responses to acute exercise for various types of skeletal muscles. For example, *GPX* activity increased the next day after running on the treadmill until exhaustion in the *m. soleus* of rats, but not in *m. tibialis* (Radák et al., 1995). In our study, the activity level of *GPX* under fatigue conditions in the F-drinking group may indicate that C_{60}FAS, by affecting endogenous antioxidants, prevents fatigue in an actively contracting muscle better than NAC and β-Alanine.

It has been proved that C_{60} fullerenes normalize cellular metabolism and nervous processes by increasing resistance to stress, increase the activity of enzymes and regenerative capacity of tissues, and also exhibit pronounced anti-inflammatory and

antiallergenic effects (Cataldo and Da Ros, 2008) and effectively regulate the ATPase activity of actomyosin (Andreichenko et al., 2013). It has been experimentally found that C_{60} fullerenes and their derivatives may be auxiliary agents in complex therapy due to their ability to intensify the protective functions of the immune and antioxidant systems of the body (Ashcroft et al., 2006; Didenko et al., 2013; Halenova et al., 2016). In this regard, the above electrophysiological and biochemical results suggest a real perspective for the use of water-soluble pristine C_{60} fullerenes as potential agents for improving the efficiency of human skeletal muscle functioning by modifying ROS-dependent mechanisms that play an important role in the development of muscle fatigue.

It was shown that under intense loads in skeletal muscles, the activity of *SOD* increases (Ji and Fu, 1992; Ji et al., 1992; Lawler et al., 1993). We investigated the isoenzyme located in the mitochondria and Mn^{2-} contained in the active center (Weisiger and Fridovich, 1973). Under the conditions of the fatiguing tests, the activity of *Mn-SOD* increased, and after application of the test substances, it decreased approximately to same level, insignificantly exceeding the control value. These results were confirmed by the data of Radák et al. (1995), where it was shown that in rats after the exhausted treadmill run, the immunoreactive content and activity of both isoenzymes of *SOD* (*Mn-SOD* and *Cu*, *Zn-SOD* are found in the cytoplasm, in erythrocytes and liver (McCord and Fridovich, 1969) increased in the *m. soleus* and *m. tibialis* immediately after the run. In animals that had been previously injected by the antioxidant, attenuation of oxidative stress was observed (i.e., a decrease in *TBARS* concentration). An increase in *Mn-SOD* was noted even in 1 day after the load in hepatic tissue (Radák et al., 1996). Additionally, it was shown that during intense swimming training, myocardial and diaphragmatic *SOD* were induced in rats (Powers et al., 1993, 1994b). Ohishi et al. (1997) showed that an adequate physical endurance load increases both the activity and the content of *Mn-SOD* and that untrained rats are very sensitive to oxidative stress during exercise. *Mn-SOD* is a reliable indicator of physical condition. It is concluded that the muscles can react to the load in such way to reduce the damage that results from the accumulation of free oxygen radicals due to increased metabolic activity.

CONCLUSION

It should be noted that there are studies in humans and animals demonstrating both various exogenous antioxidants and submaximal long-term training. The training increases the activity of endogenous antioxidants (Powers et al., 1993, 1994a) and the antioxidant defense system of glutathione (Banerjee et al., 2003) in skeletal muscles, which reduces the risk of cell damage, improving performance, and slowing down the muscle fatigue. Recent studies show that the addition of some antioxidant nutrients is necessary for physically active people (Sen, 1995; Powers et al., 1999; Banerjee et al., 2003).

The results obtained in this study confirm previous data on the mechanism of action of C_{60}FAS intramuscular injections (Prylutskyy et al., 2017). A comparative analysis was produced using a non-invasive method for C_{60}FAS administration, demonstrating a similarity of action of C_{60}FAS with effects of already known exogenous antioxidants NAC and β-Alanine. But due to its membranotropic activity and the lack of acute *in vivo* toxicity (at least at low physiological concentrations), C_{60} fullerene has a greater effective influence on the homeostasis of muscle tissue in rats, which maintains the normal physiological state of the muscle and increases the duration of its active work.

AUTHOR CONTRIBUTIONS

IV, NB, and AM designed and performed the experiments, and the *in vitro* assays were performed by OG. UR and YP were responsible for C_{60}FAS creation and characterization. WM and TT helped with preparation of the manuscript and provided funding support. DN and IM helped to collect and analyzed the data. YP and AK provided supervision and guidance throughout this study. All authors revised the manuscript for important intellectual content and approved the final version of the article.

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Protective Effect of Water-Soluble C_{60} Fullerene Nanoparticles on the Ischemia-Reperfusion Injury of the Muscle Soleus in Rats

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Abstract: The biomechanical parameters of muscle soleus contraction in rats and their blood biochemical indicators after the intramuscular administration of water-soluble C_{60} fullerene at doses of 0.5, 1, and 2 mg/kg 1 h before the onset of muscle ischemia were investigated. In particular, changes in the contraction force of the ischemic muscle soleus, the integrated power of the muscle, the time to achieve the maximum force response, the dynamics of fatigue processes, and the parameters of the transition from dentate to smooth tetanus, levels of creatinine, creatine kinase, lactate and lactate dehydrogenase, and parameters of prooxidant–antioxidant balance (thiobarbituric acid reactive substances, hydrogen peroxide, and reduced glutathione and catalase) were analyzed. The positive therapeutic changes in the studied biomechanical and biochemical markers were revealed, which indicate the possibility of using water-soluble C_{60} fullerenes as effective prophylactic nanoagents to reduce the severity of pathological conditions of the muscular system caused by ischemic damage to skeletal muscles.

Keywords: C_{60} fullerene; muscle soleus of rat; ischemia; biomechanical and biochemical parameters

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1. Introduction

Among the muscle pathologies that develop in skeletal muscles in various injuries, ischemic injuries account for more than 35% of the total number of injuries to the musculoskeletal system. Ischemic reperfusion injuries of skeletal muscles after acute arterial occlusion, in many cases, are the cause of severe pathologies and mortality [1]. Ischemic tissue damage is a cascade of biochemical reactions that are initiated under conditions of hypoxia after a few minutes of ischemia as a result of insufficient blood supply [2]. The ischemic cascade usually continues for 2–3 h after ischemia, but can last for several days, even after normal blood flow has been restored [3]. At the same time, with ischemia lasting 3 h or more, both muscle necrotic changes and nervous degradation occur. The amount of necrosis in the muscle tissue can be up to 60% [4]. In addition, with ischemic reperfusion, the expression of adhesive molecules on the endothelium is increased. Activated neutrophils attracted to the site of injury release free radicals [2]. The last ones provoke vasoconstriction, which is a characteristic manifestation of ischemic damage. In addition, ischemia—reperfusion injury of skeletal muscles is one of the main causes of post-traumatic pathologies after surgical procedures [5,6]. The main goal in the treatment of muscle ischemia is the rapid restoration of blood flow in the damaged areas. However, such therapy

often leads to a new pathophysiological process-reperfusion injury, which can also cause significant damage to the muscle tissue. The rapid establishment of the severity of ischemic injury is critical for further therapy; however, there are currently no accurate diagnostic tests to achieve this goal [7]. Literature data indicate that during reperfusion, free radicals, together with calcium activated caspases and calpains, can lead to apoptosis and damage to the DNA and mitochondria, resulting in additional loss of muscle functions [8,9]. So, the interaction of the hydroxyl radical with the hydrogen atoms of the methyl groups of polyunsaturated fatty acids initiates the peroxidation of the membrane lipids, which in turn leads to increased permeability of the cell membranes [2].

It is known that C_{60} fullerenes efficiently capture and inhibit free radicals in in vivo and in vitro systems [10–12]. Whether the double chemical bonds in the structure of C_{60} fullerene are electron-deficient determines its ability to attach up to six electrons [13]. In our previous work, it was shown that the administration of biocompatible water-soluble C_{60} fullerenes [14] after the initiation of ischemic damage to the skeletal muscle leads to a significant positive therapeutic effect [15]. At the same time, it was revealed that the administration of C_{60} fullerenes directly into the damaged muscle complicates their steady distribution over the tissues and, thus, reduces the antioxidant effect of the drug. In this case, the time elapsed after the initiation of ischemia before the administration of the therapeutic drug is of great importance, as the beginning of the ischemic cascade of muscle tissue damage occurs already in the first seconds after reperfusion [16]. All of this served as the basis for further investigation of the effect of C_{60} fullerene aqueous solution (C_{60}FAS) on the dynamics of the contractile process of muscle soleus in rats against the background of ischemic pathology when administered intramuscularly 1 h before the initiation of ischemia, depending on the dose (protective effect).

2. Results and Discussion

2.1. Characterization of C_{60}FAS

The monitoring of the C_{60} fullerene morphology in an aqueous solution is important for controlling the particle size distribution profile, which may influence the C_{60}FAS bioactivity and toxicity [17–20]. The prepared C_{60}FAS was characterized by atomic force microscopy (AFM) and scanning tunneling microscopy (STM).

The study of the C_{60} fullerene films deposited from an aqueous solution revealed a high degree of molecule dispersion in the solution. It turned out that the prepared C_{60}FAS contained both single C_{60} fullerene and its labile nanoaggregates with a size of 1.3–35 nm. The majority of C_{60} molecules were located chaotically and separately along the surface (see the objects with a height of ~0.7 nm in Figure 1), or in the form of bulk clusters consisting of several tens of C_{60} molecules [21] (objects with a height of 1.3–2 nm in Figure 1. Such an arrangement of C_{60} molecules formed because of electrostatic repulsion between them; the zeta potential value was −25.3 mV at room temperature [22], indicating a high solute stabilization.

2.2. Biomechanics of Injured Muscle Contractions

After the initiation of ischemic damage, the contraction force of the rat muscle soleus, caused by 6 s non-relaxation stimulation pools, decreased to 28 ± 2% of the control values at the first contraction and to 9 ± 1% at the tenth (Figure 2). The decrease in the integrated power of the muscle contraction was 39 ± 2% of the control values at the first contraction and 6 ± 2% at the tenth, respectively. The time to reach the maximum force response increased from 451 ± 5 ms at the first contraction to 978 ± 7 ms at the tenth. Thus, a sharp decrease in the force activity of the muscle was observed at the first contractions with a progressive decrease in biomechanical parameters. This confirms the literature data that in the process of ischemia−reperfusion, a significant decrease in the force of the contraction of skeletal muscle occurs. The progressive decrease in the force response lasts at least 5 days, after which the recovery process takes place [23,24].

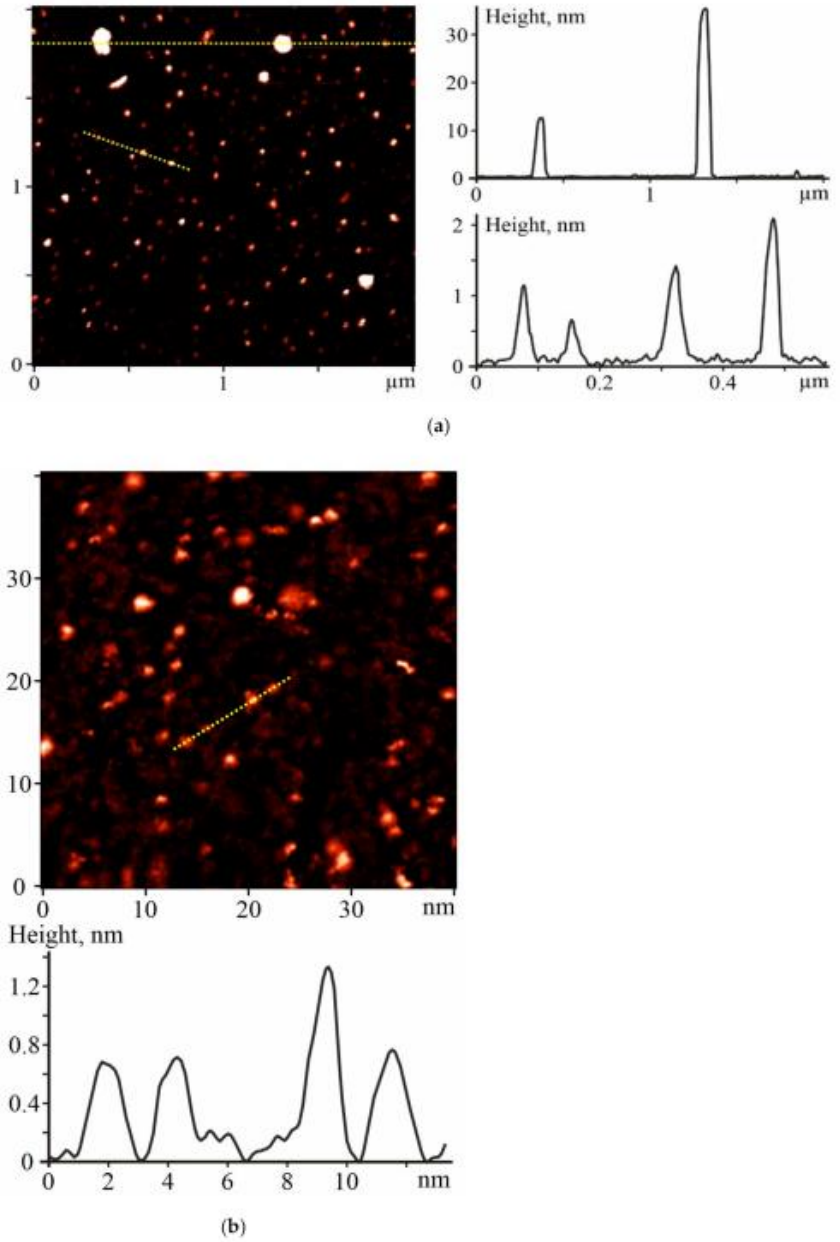

Figure 1. (**a**) Atomic force microscopy (AFM) and (**b**) scanning tunneling microscopy (STM) images of the C_{60} fullerene nanoparticles on the mica and gold surfaces, respectively, and their profiles along the marked lines. C_{60} fullerenes were precipitated from C_{60}FAS with a 0.15 mg/mL concentration.

The use of C_{60}FAS injections increased the muscle force response as follows: at a dose of 0.5 mg/kg of C_{60}FAS, 58 ± 1% and 51 ± 1% of the control values on the first and tenth contractions, respectively; at a dose of 1 mg/kg of C_{60}FAS, 78 ± 2% and 56 ± 2%, respectively; and at a dose of 2 mg/kg of C_{60}FAS, 79 ± 1% and 58 ± 1%, respectively. At a dose of 0.5 mg/kg of C_{60}FAS, the integrated power of the muscle contraction was 54 ± 2% of the control values at the first contraction and 52 ± 2% at the tenth, respectively. After increasing the doses of C_{60}FAS, this parameter was 76 ± 1% and 55 ± 1% at 1 mg/kg and

78 ± 2% and 59 ± 2% at 2 mg/kg, respectively. The time to reach the maximum force response increased from 373 ± 3 ms at the first contraction to 755 ± 6 ms at the tenth at a dose of 0.5 mg/kg C_{60}FAS; from 343 ± 4 ms at the first contraction to 457 ± 6 ms at the tenth at a dose of 1 mg/kg of C_{60}FAS; and from 291 ± 5 ms at the first contraction to 399 ± 7 ms at the tenth at a dose of 2 mg/kg of C_{60}FAS.

It is important to note that after the administration of C_{60}FAS, the force response of the ischemic muscle did not decrease by more than 50% of the control values, even with the tenth act of contraction. At the same time, the C_{60}FAS dose increasing from 1 to 2 mg/kg did not lead to significant therapeutic effects. Thus, the data obtained indicate a significant positive trend in the use of C_{60}FAS for prophylactic purposes. Based on the data obtained, it can be concluded that pretraumatic administration of C_{60}FAS at a dose of 1 mg/kg reduces the severity of ischemic damage in the muscle by 60–75%. A decrease in the C_{60}FAS dose leads to a decrease in the therapeutic effect, while its increase does not lead to a significant increase in the biomechanical parameters. In addition, it should be noted that the use of C_{60} fullerene therapy did not eliminate the developing fatigue processes in the ischemic muscle; the integrated muscle power decreased with each subsequent pool of the stimulation signal. Therefore, the next stage of the study was to investigate the nature of the muscle response during prolonged fatigue stimulation. At this stage, we applied only one, the most optimal, dose of C_{60}FAS of 1 mg/kg.

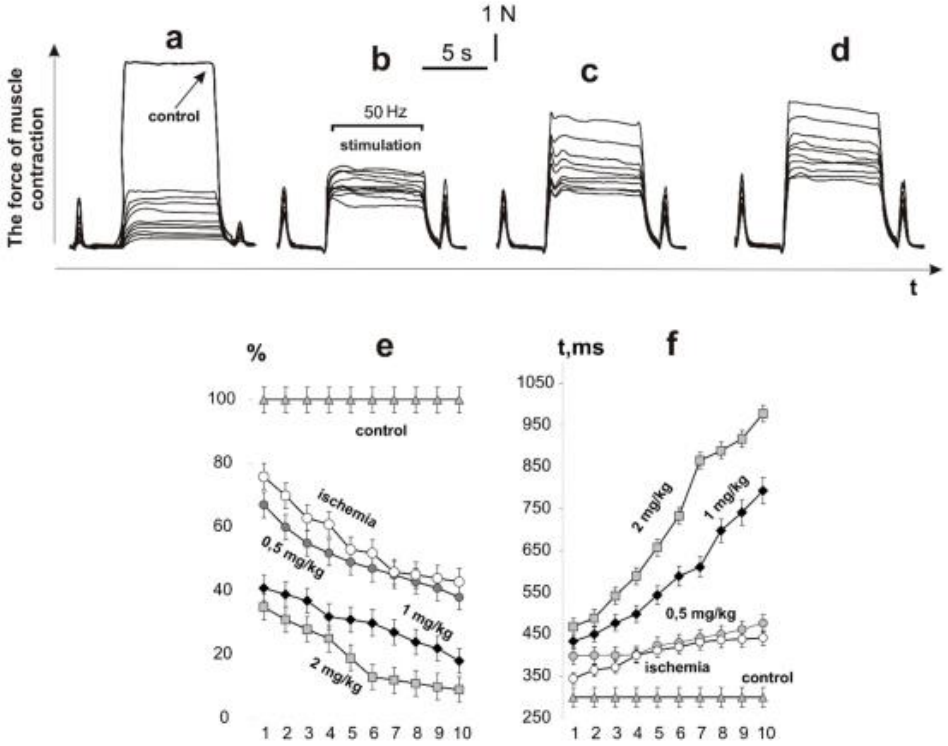

Figure 2. The force of contraction of the rat muscle soleus, caused by 10 (indicated 1,2, . . . , 10) consecutive 6 s non-relaxation pools of stimulation: ischemic muscle without C_{60}FAS (control: native muscle) (**a**); administration of C_{60}FAS 1 h before muscle ischemia at doses of 0.5 (**b**), 1 (**c**), and 2 mg/kg (**d**). Integrated muscle power, calculated area under the force curve, as a percentage of control values (**e**). Time to reach the maximum force response (**f**).

It has been shown that ischemia−reperfusion increases the degree of fatigue processes development and reduces the force of muscle contraction to 40% after 1 h of ischemia and to 70% after 3 h. Recovery of the muscle force response was observed only at the

end of the second week after ischemia−reperfusion [6]. Registration of the contraction force of the ischemic muscle soleus of a rat with 1 Hz stimulation for 1800 s (Figure 3b,c) revealed a decrease in the integrated muscle power (Figure 3d), which was 35 ± 4% of the control value. Intramuscular injections of C_{60}FAS changed this parameter to 67 ± 4%. The time for the decrease in the force response by 50% and 25% from the initial values was 940 ± 11 s and 1580 ± 18 s, respectively, without C_{60} fullerene therapy, and 1430 ± 17 s and 1690 ± 14 s, respectively, with the administration of C_{60}FAS (Figure 3e). The maximum and minimum recorded contraction forces of the ischemic muscle throughout the entire duration of stimulation were 1.8 ± 0.3 N (3.1 ± 0.4 N in control) and 0.18 ± 0.01 N (2.9 ± 0.4 N in control), respectively (Figure 3f). When C_{60}FAS was injected, this indicator was 2.5 ± 0.4 N and 0.6 ± 0.1 N, respectively, which shows its 52% therapeutic effect at the stages of maintaining maximum force responses during the development of fatigue processes.

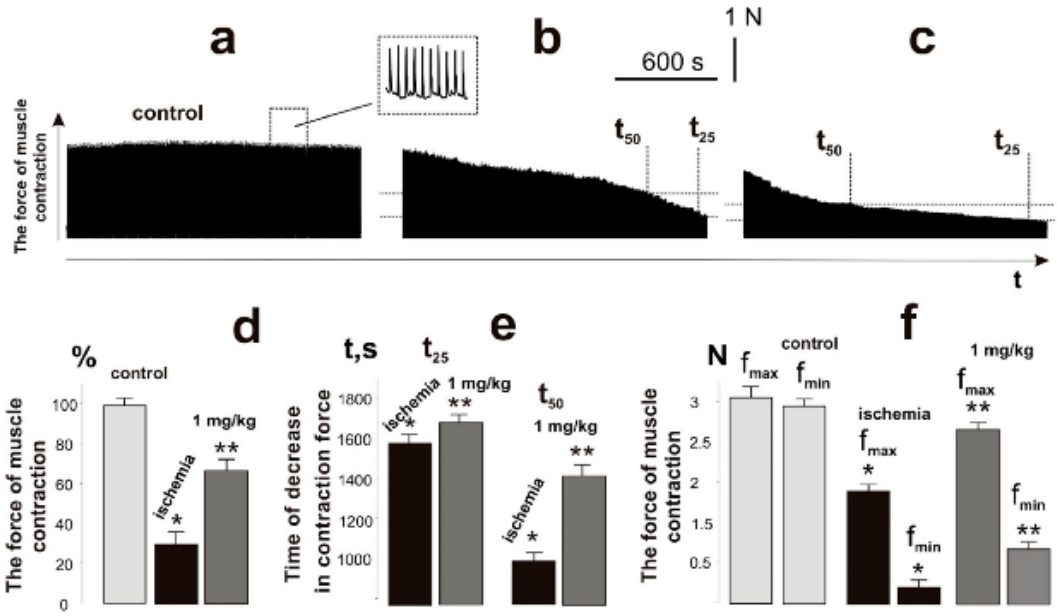

Figure 3. Registration of the force of contraction of the muscle soleus of the rat with the use of 1 Hz stimulation with a duration of 1800 s: control, native muscle (**a**); ischemic muscle without the administration of C_{60}FAS (**b**); administration of C_{60}FAS (1 mg/kg) 1 h before muscle ischemia (**c**); integrated muscle power, presented as a percentage of the control values (**d**); the time of the decrease in the force response by 50% and 25% of the initial values (t_{50} and t_{25}) (**e**); and maximum (f_{max}) and minimum (f_{min}) fixed forces of muscle contraction throughout the entire duration of stimulation (**f**). * $p < 0.05$ relative to the control group; ** $p < 0.05$ relative to the ischemia group.

In the process of skeletal muscle functioning, the most important quality indicator of its work is the rate of occurrence of smooth tetanic contraction (a state of continuous muscle tension after complete summation of single contractions). Even minimal changes in the structure of the impulses generated by motor neurons, damage to myocyte membranes, development of the inflammatory process, changes in muscle stiffness, electrical properties of membranes, and the duration of hyperpolarization significantly change the time of occurrence of smooth tetanic contractions [25,26]. In addition, during muscle activity, its individual motor units generate non-fused tetanic contractions, which are characterized by variable strength and varying degrees of fusion. The synchronization of this process depends on many factors and is also a vulnerable link in the development of pathological processes in the muscle [27,28]. Therefore, the next stage of the study was to investigate the

biomechanical markers of the appearance of smooth tetanic contractions in the ischemic muscle soleus of the rat.

The smooth tetanic contractions (maximum force response) appeared in 3450 ± 12 ms and reached 70 ± 8 mN after using stimulation pools of increasing frequency (Figure 4). The ischemically damaged muscle throughout the entire stimulation pool did not reach the stage of smooth tetanic contraction. The maximum force of a single contraction increased from 24 ± 2 mN to 37 ± 3 mN. The minimum value of the force response in one spike of the dentate tetanus decreased to 12 ± 1 mN. It should be noted that a decrease in this parameter to zero leads to the appearance of smooth tetanus. Preliminary injections of C_{60}FAS changed the biomechanical parameters of ischemized muscle soleus transition from dentate to smooth tetanus, which appeared after 4950 ± 32 ms and reached 58 ± 2 mN. It should be noted that the injection of C_{60}FAS eliminated both the abrupt decrease in the force of contraction and the fluctuation component of the contractile process. Thus, the preventive effect of C_{60}FAS injection on the biomechanical parameters of the transition of ischemic muscle from dentate to smooth tetanus was 68 ± 4% of the control values.

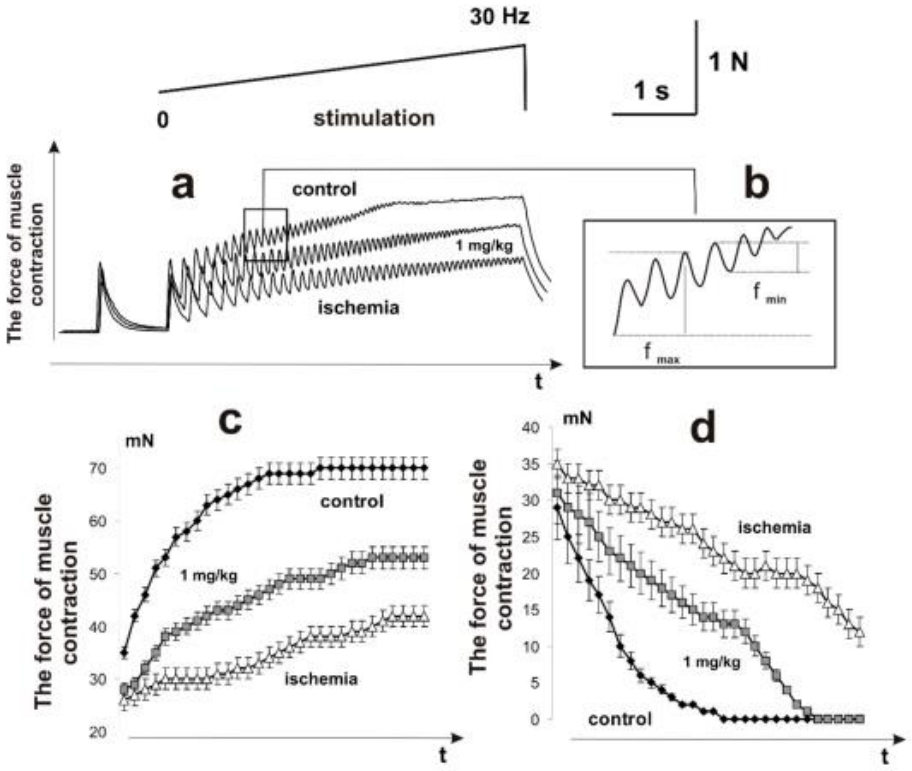

Figure 4. Biomechanical parameters of muscle soleus transition from dentate to smooth tetanus after using increasing stimulation with a maximum frequency of 30 Hz for 6 s: mechanograms of the native muscle contraction, control (**a**); f_{max} is the maximum force of a single contraction, f_{min} is the minimum value of the force response in one spike of the dentate tetanus (a decrease in this parameter to zero leads to the appearance of smooth tetanus) (**b**); and changes in the parameters f_{max} (**c**) and f_{min} (**d**) for each of the single contractions before the transition of the force response to smooth tetanus when an increasing stimulation signal is applied.

2.3. Blood Biochemical Indicators of Rats with Injured Muscle

The analysis of the biochemical composition of the blood of rats during the development of ischemia−reperfusion reflected the changes occurring in the damaged skeletal muscle and made it possible to evaluate the therapeutic effect of the applied drug on the

pathological process. The biochemical indicators of the development of fatigue processes selected by us for the study, such as creatinine, lactate dehydrogenase (LDH), lactate (LA), and creatine kinase (CK) are also indicators of physiological disorders in the muscle tissue due to the development of ischemic damage (Figure 5).

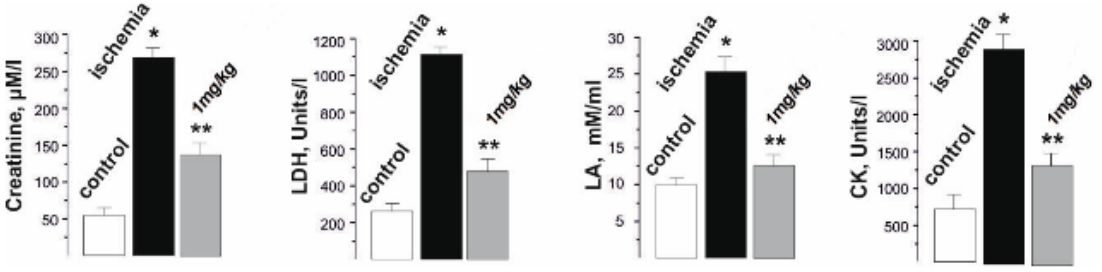

Figure 5. Biochemical indicators (creatinine, lactate dehydrogenase (LDH), lactate (LA), and creatine kinase (CK)) in the blood of rats after 1 Hz stimulation of the ischemic muscle soleus for 1800 s. * $p < 0.05$ relative to the control group; ** $p < 0.05$ relative to the ischemia group.

The change in the level of creatinine, a product formed in the muscles during the destruction of intramuscular structures, made it possible to assess the level of damage to the myocytes during prolonged contractions. This indicator increased from 50 ± 2 μM/L in the control to 25,750 ± 51 μM/L after muscle ischemia. The administration of C_{60}FAS prior to muscle ischemia reduced this indicator to 122 ± 2 μM/L. In our opinion, the decrease in the creatinine fraction was due to the C_{60} molecules that protect the membranes of skeletal muscle cells from nonspecific free radical destruction, effectively absorbing the reactive oxygen species (ROS).

The level of changes in LDH, an enzyme that generates lactic acid, made it possible to assess the muscle performance after ischemia. The change in the level of this enzyme from 220 ± 8 units/l in the control to 1115 ± 22 units/l after ischemia is evidence of the development of significant muscle dysfunctions associated with the development of the inflammatory process. An increase in the LDH fraction in the blood is the result of both the physiological destruction of the myocyte walls caused by their performance [29] and an increase in LA content during prolonged muscle activation. Preliminary administration of C_{60}FAS reduced the LDH level to 442 ± 11 units/l. A decrease in this enzyme upon the administration of C_{60}FAS may indicate both a decrease in mechanical damage to muscle fibers and in LA concentration in the muscular system in general.

In active muscle, most metabolic and biochemical processes occur under anaerobic conditions; the muscle uses a significant amount of mitochondrial enzymes and, as a result, a large amount of LA accumulates in it, which cannot be oxidized during prolonged muscle stimulation. An increase in the level of lactic acid in active muscle indicates that the level of its entry into the cell exceeds the level of its oxidation and excretion. In the control values, the LA level was 11 ± 2 mM/mL. After ishimization, its value increased to 27 ± 3 mM/mL. C_{60}FAS injections reduced the LA level to 17 ± 1 mM/mL. Thus, pre-C_{60} fullerene therapy led to a decrease in the LA level by almost 50%.

CK is an enzyme found in high concentrations in the skeletal muscle. The release of this enzyme from the cells and, accordingly, an increase in CK activity in the blood are observed after mechanical damage to the muscles. The increase in the CK fraction in the blood during the induction of ischemia from 560 ± 13 units/l in the control to 2830 ± 22 units/l is the result of the rapid physiological destruction of the myocyte walls, which intensifies during active prolonged non-relaxation muscle contraction. The CK level decreased significantly (more than three times) and reached 820 ± 23 units/l after the application of C_{60}FAS. CK is an enzyme from the energy supply system of musculoskeletal cells that catalyzes the transfer of a phosphate group from ATP to a creatine molecule with the formation of a high-energy compound creatine phosphate, which is used by the body

as an energy substance when physical activity increases. A change in its concentration is one of the known markers of the pathological processes in the muscle and characterizes the depletion of the cell's energy reserves. So, it was shown that during 3 h of ischemia-reperfusion of muscle soleus the depletion of ATP reserves was about 95%, and glycogen was depleted by 88% [6]. From a functional point of view, these data indicate that a large amount of high-energy phosphate compounds is consumed by an ischemic-damaged muscle cell so as to maintain homeostasis and, as a consequence, metabolic disorders occur, leading to a significant increase in ischemic muscle fatigue. Thus, preliminary injections of C_{60}FAS significantly increase the energy capabilities of actively contracting ischemic muscle.

The pathological inflammatory processes that occur immediately after ischemia-reperfusion are a source of ROS and contribute to the intensification of lipid peroxidation processes [8]. This interferes with the adequate performance of muscle work and significantly increases the duration of the recovery period. During reperfusion, oxygen entering the tissues initiates the oxidation of xanthine and hypoxanthine by xanthine oxidase, which leads to the formation of a large amount of superoxide anion radical and hydrogen peroxide. Hydrogen peroxide is converted to hydroxyl radicals by the reduction of metal ions. Mitochondria damaged by ischemia can produce more electrons because of their "leakage" from the electron transport chain. These electrons are involved in the formation of the superoxide radical anion. In addition, during ischemia−reperfusion, the expression of adhesive molecules on the endothelium increases. Activated neutrophils attracted to the site of injury also release free radicals and provoke vasoconstriction, which is a characteristic manifestation of ischemic damage [2–5]. As a result of biochemical tests, the increased level of peroxidation markers and oxidative stress (catalase (CAT), hydrogen peroxide (H_2O_2), and thiobarbituric acid reactive substances (TBARS), and the reduced glutathione (GSH)) after muscle ischemia, as well as their significant decrease after C_{60}FAS injections before muscle ischemia (Figure 6), were revealed.

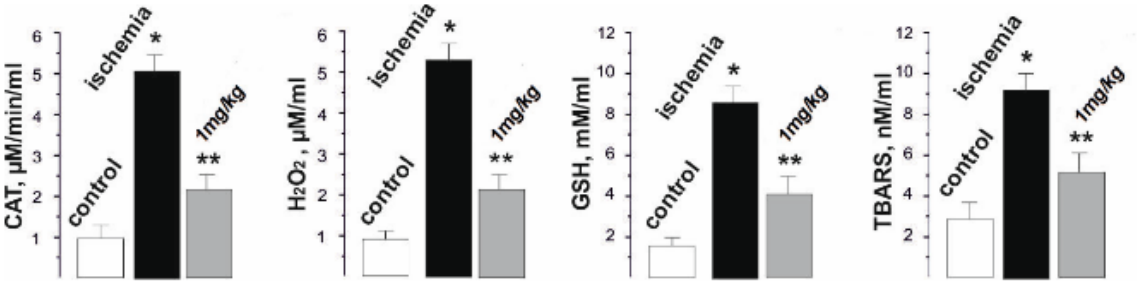

Figure 6. Levels of catalase (CAT), hydrogen peroxide (H_2O_2), and thiobarbituric acid reactive substances (TBARS) in rat blood after 1 Hz stimulation of the ischemic muscle soleus for 1800 s. * $p < 0.05$ relative to the control group; ** $p < 0.05$ relative to the ischemia group.

So, the CAT activity increased from 0.9 ± 0.1 μM/min/mL in the control to 5.1 ± 0.3 μM/min/mL after muscle ischemia, and decreased to 2.1 ± 0.1 μM/min/mL with C_{60} fullerene therapy. The H_2O_2 level was 5.4 ± 0.4 μM/mL during ischemia (0.8 ± 0.1 μM/mL in the control) and 2.2 ± 0.2 μM/mL after the administration of C_{60}FAS. The GSH concentration was 8.3 ± 0.6 mM/mL with ischemia (1.8 ± 0.1 mM/mL in the control) and 3.9 ± 0.2 mM/mL with C_{60}FAS injection. Finally, the TBARS level was 9.8 ± 1.0 nM/mL with ischemia (2.3 ± 0.2 nM/mL in the control) and 5.8 ± 0.5 nM/mL with the administration of C_{60}FAS.

Thus, there is a clear tendency towards a decrease in the described biochemical parameters by about 45–60% with the prophylactic use of C_{60}FAS. We suppose that C_{60} fullerenes can affect the activity of endogenous antioxidants, preventing the onset of

dysfunction in the active muscle and, thus, maintaining it within the physiological norm during the entire process of its contraction.

In summary, oxidative stress causes cellular damage in ischemic pathology. The mediators of oxidative stress are ROS, including superoxide anion radical, hydroxyl radical, singlet oxygen, and hydrogen peroxide, which damage cellular targets such as DNA, proteins, and lipids [30]. After ischemia, a sequential chain of pathophysiological cascades occurs, including massive intracellular release of Ca^{2+}, disruption of the mitochondrial electron transport chain, release of neutrophils, acute inflammatory reactions, and the formation of free radicals, which, in turn, enhance apoptotic or necrotic cell death. The endogenous antioxidant defense system of the body, at the beginning of the development of the ischemic cascade, can neutralize only a small amount of ROS by enzymatic and non-enzymatic pathways [31].

The chemical structure of C_{60} fullerene with an abundance of conjugated double bonds and low-lying lower unoccupied molecular orbitals makes it very susceptible to free radicals. Thanks to this, C_{60} fullerene can react with many ROS without losing its antioxidant properties [32]. The protective effect of C_{60} fullerene on the absorption of superoxide anions does not lead to an increased production of hydrogen peroxide [33]. C_{60} fullerene promotes cell survival by altering the cellular redox state and enzyme activity [34]. C_{60} fullerene reduces lipid peroxidation by actively absorbing ROS [35]. C_{60} fullerenes can penetrate the cell membrane and localize in the mitochondria [36,37], which are the source of ROS during the development of ischemic cell damage. Finally, the obtained above results are also confirmed by the previously obtained data on the effect of water-soluble C_{60} fullerenes on the functions of the antioxidant systems of the body in inflammatory and pathological processes [38–41]. They indicate that the development of medical nanotechnology based on water-soluble C_{60} fullerenes, considering their powerful antioxidant properties, opens up new possibilities in the treatment and prevention of ischemic damage to skeletal muscles.

3. Materials and Methods

To obtain C_{60}FAS, a method was used that is based on the transfer of C_{60} molecules from toluene to water, followed by sonication [42,43]. Briefly, a saturated solution of pure C_{60} fullerene in toluene (purity >99.5%), where its concentration corresponds to a maximum solubility of ~2.9 mg/mL, was mixed with the same volume of distillate in an open beaker. The formed aqueous phases was subjected to ultrasound (frequency 8 Hz, duration 8 h). The obtained C_{60}FAS at the maximum concentration of C_{60} fullerene 0.15 mg/mL remained stable for 18 months at a temperature of +4 °C.

AFM and STM were performed to determine the size of the C_{60} fullerene particles in aqueous solution. Measurements were done with the "Solver Pro M" system (NT-MDT, Moscow, Russia). A drop of investigated solution was transferred on the atomic-smooth substrate to deposit layers. Measurements were carried out after complete evaporation of the solvent. For AFM studies, a freshly broken surface of mica (SPI supplies, V-1 grade) was used as a substrate. Measurements were carried out in a semicontact (tapping) mode with AFM probes of the RTPESPA150 (Bruker, Billerica, MA, 6 N/m, 150 kHz) type. STM studies were performed with the Au (111) surface obtained after annealing the substrates of Au/mica (Phasis, Switzerland) in a gas burner flame (propane−butane). The typical tunneling current and voltage values were 0.027–0.1 nA and 0.1–1 V, respectively.

The experiments were performed on male Wistar rats aged 3 months, weighing 170 ± 5 g. The study protocol was approved by the bioethics committee of 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 № 3446—IV 21.02.2006, Kyiv, on the Protection of Animals from Cruelty during medical and biological research.

Fifty rats divided into five groups (10 animals each) were used in the study—the control group (native muscle; $n = 10$); the ischemia group without C_{60}FAS administration (ischemic muscle; $n = 10$); and the group where C_{60}FAS was administered once intramuscularly 1 h before muscle ischemia at doses of 0.5 ($n = 10$), 1 ($n = 10$), and 2 mg/kg ($n = 10$), respectively.

It should be emphasized that during the experiments, the control group of ischemic animals received injections of saline with the same dose as C_{60}FAS (1 mg/kg; $n = 10$). However, the results obtained did not reveal significant differences in the studied biomechanical and biochemical parameters in this group and in the group of ischemic animals without C_{60}FAS administration. It is also important to note that, in accordance with our previous study, the maximum tolerated dose of C_{60}FAS was 721 mg/kg for i.p. administration to mice [22].

Anesthesia of the animals was performed by the intraperitoneal administration of nembutal (40 mg/kg). Standard preparation of the experiment also included the cannulation (*a.* carotis communis sinistra) for the therapeutic administration of the drug and pressure measurement, tracheotomy, and laminectomy at the lumbar spinal cord level. For muscle ischemia, the branch of the femoral artery of the animal, which provides blood supply of the experimental muscle, was dragged by ligatures. The duration of ischemia was 3 h. Muscle soleus of the rats were released from the surrounding tissues and their tendons were cut across in a distal part. The ventral roots were cut in places of their exit from the spinal cord for the modulated stimulation of efferents in L4–L5 segments. Filaments of the ventral roots were cut and fixed on stimulating electrodes, and a special device was used for cyclic sequence distribution of electrical signals via the filaments. Stimulation of the efferents was performed by electric impulses with a frequency of 1 to 50 Hz, and the duration of each pulse was 2 ms, formed by using a pulse generator. A control of the external load on the muscle was carried out with the help of an original mechanical stimulator [44]. In the process of analyzing the obtained results, the next parameter was used, namely the integrated muscle power (calculated area under the force curve), which is an indicator of the general performance of the muscle with the applied stimulation pools. The development of the muscle contractile activity was assessed using the method of calculating time intervals when 50% and 25% of the levels of force responses were reached during stimulation.

The level of enzyme content in the blood of the experimental animals (creatinine, LDH, LA, CK, TBARS, H_2O_2, GSH, and CAT), as markers of muscle injury [45,46], was determined using clinical diagnostic equipment, namely a haemoanalyzer [15].

Statistical processing of the results was performed using methods of variation statistics using software Original 9.4. At least six repetitions for each measurement were conducted. Data are expressed as the means ± SEM for each group. The differences among the experimental groups were detected by one-way ANOVA followed by Bonferroni's multiple comparison test. Values of $p < 0.05$ were considered significant.

4. Conclusions

Thus, it was shown that the pretraumatic administration of water-soluble C_{60} fullerenes (nanoparticles with size of 0.7–35 nm) at a dose of 1 mg/kg reduces the severity of ischemic damage in the rat muscle soleus by 60–75%. In particular, intramuscular injection of C_{60}FAS produces a 52% therapeutic effect at the stages of maintaining maximum force responses during the development of fatigue processes. The preventive effect of C_{60}FAS injections on the biomechanical parameters of the transition of ischemic muscle from dentate to smooth tetanus is about 68% of the control values. Finally, the administration of C_{60}FAS before muscle ischemia significantly reduced the blood biochemical parameters of the rat (by about 45–60%), which indicates the promise of its use for prophylactic purposes.

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Analysis of Biomechanical Parameters of Muscle Soleus Contraction and Blood Biochemical Parameters in Rat with Chronic Glyphosate Intoxication and Therapeutic Use of C_{60} Fullerene

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Abstract: The widespread use of glyphosate as a herbicide in agriculture can lead to the presence of its residues and metabolites in food for human consumption and thus pose a threat to human health. It has been found that glyphosate reduces energy metabolism in the brain, its amount increases in white muscle fibers. At the same time, the effect of chronic use of glyphosate on the dynamic properties of skeletal muscles remains practically unexplored. The selected biomechanical parameters (the integrated power of muscle contraction, the time of reaching the muscle contraction force its maximum value and the reduction of the force response by 50% and 25% of the initial values during stimulation) of muscle soleus contraction in rats, as well as blood biochemical parameters (the levels of creatinine, creatine phosphokinase, lactate, lactate dehydrogenase, thiobarbituric acid reactive substances, hydrogen peroxide, reduced glutathione and catalase) were analyzed after chronic glyphosate intoxication (oral administration at a dose of 10 μg/kg of animal weight) for 30 days. Water-soluble C_{60} fullerene, as a poweful antioxidant, was used as a therapeutic nanoagent throughout the entire period of intoxication with the above herbicide (oral administration at doses of 0.5 or 1 mg/kg). The data obtained show that the introduction of C_{60} fullerene at a dose of 0.5 mg/kg reduces the degree of pathological changes by 40–45%. Increasing the dose of C_{60} fullerene to 1 mg/kg increases the therapeutic effect by 55–65%, normalizing the studied biomechanical and biochemical parameters. Thus, C_{60} fullerenes can be effective nanotherapeutics in the treatment of glyphosate-based herbicide poisoning.

Keywords: glyphosate; C_{60} fullerene; muscle soleus of rat; biomechanical parameters; blood biochemical parameters

1. Introduction

Glyphosate (N-(phosphonomethyl) glycine) is a non-selective herbicide most commonly used for weed control. Among herbicides, it ranks first in the world in production. Many agricultural crops are genetically engineered to tolerate glyphosate. This significantly increases the effectiveness of weed control in these crops. The effect of glyphosate on a plant is due to the fact that it inhibits the components of the enzyme

system of the shikimate pathway of biosynthesis of benzoic aromatic compounds [1]. Animals do not have such an enzyme system and therefore this herbicide is considered to be relatively harmless to them. The half-lethal dose (LD_{50}) of glyphosate is >5 kg/kg of rat body weight with a single administration [1].

In recent years, there has been a growing worldwide concern about the possible direct and indirect health effects of the widespread use of glyphosate. In 2015, the World Health Organization reclassified glyphosate as likely carcinogenic to humans [2]. There is considerable controversy regarding its carcinogenicity and toxicity, with very different opinions of the scientists and regulatory bodies involved in the glyphosate study. One of the key aspects of this controversy is the extent of pathological changes in laboratory animals that are caused by glyphosate [3]. The most convincing data indicate that glyphosate causes hemangiosarcomas, tumors and malignant lymphomas, renal and liver adenomas, nervous and miotic disorders of a wide spectrum of severity [3].

Glyphosate also causes numerous morphological, physiological and biochemical disorders in the cells and organs of animals, including mammals. It worsens the condition of the gastrointestinal tract: a violation of its contractile function is observed already at a concentration of 3 mg/L; the violation of motility continues after the removal of glyphosate from the incubation solution [4]. The use of glyphosate as a herbicide in agriculture can lead to the presence of its residues and metabolites (aminomethylphosphonic acid) in food for human consumption and thus pose a threat to human health. The authors [5] found that glyphosate reduces energy metabolism in the brain, its amount increases in white muscle fibers.

At the same time, the effect of chronic use of glyphosate on the dynamic properties of skeletal muscles remains practically unexplored. It was shown that the activity of acetylcholinesterase (AChE) did not change in the muscles and brain of animals exposed to glyphosate during the first 96 h. On the contrary, the expression of this enzyme in muscle tissue changed [6]. The consequence of these pathological processes is disorders in the dynamics of contraction of the muscular system of varying severity. The results [7] show that glyphosate intoxication increases energy expenditure to maintain homeostasis. In particular, there was a decrease in the level of glycogen and triglycerides in all organs and an increase in lipid peroxidation (LPO).

The mechanisms for the toxicity of glyphosate-based drugs are complex. It is difficult to separate the toxicity of glyphosate from the toxicity of the drug as a whole, or to determine the contribution of surfactants to the overall toxicity. As a result, the treatment of poisoning occurs for a long time, symptomatic and ineffective [8].

Studies of the effect of a glyphosate-based herbicide on AChE enzyme activity and oxidative stress at concentrations of 0.5–10.0 mg/L for 96 h followed by an equal recovery period indicate the presence of LPO and AChE inhibition. The results also showed an increased level of thiobarbituric acid reactive substances (TBARS) at all tested herbicide concentrations, which remained elevated even after a recovery period [9]. According to the authors, the triggering mechanisms of the onset of these pathological cascades are associated precisely with the formation of a large number of free radicals. They initiate LPO, cause direct inhibition of mitochondrial enzymes of the respiratory chain and their ATPase activity, inactivation of glyceraldehyde 3-phosphate dehydrogenase and membrane sodium channels.

The ability of C_{60} fullerenes to inactivate free radicals was described back in 1991 [10]. One C_{60} molecule simultaneously captures 34 methyl radicals, effectively inactivates the superoxide anion radical and hydroxyl radicals in vitro system, protecting cell membranes from oxidation [11]. It is assumed that biocompatible and water-soluble C_{60} fullerenes [12] can be considered as powerful scavengers of free radicals during the development of ischemia and fatigue processes in skeletal muscle [13,14]. In our previous works on in vivo models, it was shown that the usage of safe doses of water-soluble C_{60} fullerene at the initiation of various pathologies leads to significant positive therapeutic

effects, in particular, during acute liver injury, colorectal cancer, obesity, acute cholangitis and hemiparkinsonism [15–20].

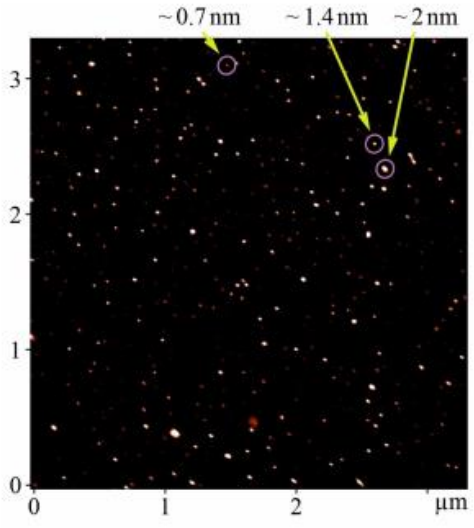
Based on the above data, the purpose of this work was to estimate the therapeutic effect of water-soluble C_{60} fullerene, as a powerful antioxidant, on the development of muscle pathologies in rat skeletal muscle caused by chronic glyphosate intoxication.

2. Results and Discussion

2.1. AFM Analysis

It is known that the size of C_{60} fullerene particles in aqueous solution strongly correlates with their specific biological properties and toxicity. So, the antibacterial activity of C_{60} fullerene is connected with its ability to undergo aggregation [21]; the macrophage apoptosis induced by aqueous C_{60} fullerene aggregates changes the mitochondrial membrane potential [22]; the respiratory toxicity and immunotoxicity of C_{60} fullerenes in mice and rats after nose inhalation strictly depends on their nano- and micro-size [23]; depending on the size C_{60} fullerenes can inhibit BK_{Ca} but not K_v channels in pulmonary artery smooth muscle cells [24], penetrate through plasma membrane inside the cell [25] or be adsorbed on the surface of the membrane [26]. Therefore, the size effect of C_{60} fullerene particles in aqueous solution is considered now to be very important.

The atomic force microscopy (AFM) study of C_{60} fullerene films deposited from an aqueous solution revealed a high degree of molecules dispersion in solution. It turned out that C_{60} fullerene aqueous solution (C_{60}FAS) contains both single C_{60} fullerene (see the objects with a height of ~0.7 nm in Figure 1a) and its labile nanoaggregates (objects with a height of 1.4–60 nm in Figure 1b). The majority of C_{60} molecules were located chaotically and separately along the surface, or in the form of bulk clusters. Thus, C_{60}FAS is a polydisperse colloid nanofluid. This result is in a good agreement with our previous probe microscopic data [27,28].



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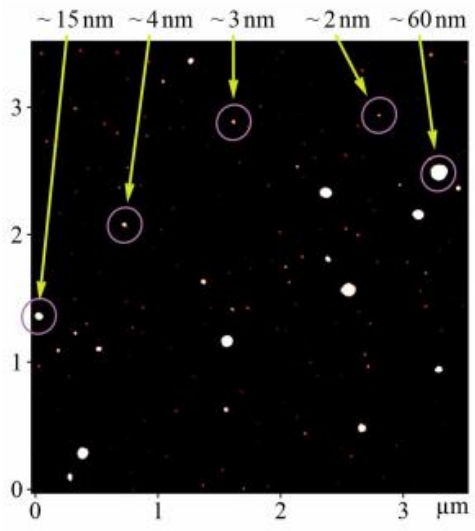

Figure 1. AFM images (tapping mode) of C_{60} fullerene nanoparticles on the mica surface (concentration 0.15 mg/mL) (**a**) objects with a height of ~0.7 nm (**b**) objects with a height of 1.4–60 nm.

In addition, the stability of the used C_{60}FAS was evaluated by the zeta potential measurement. This value was shown to be −30.3 mV at room temperature. Such a high (by absolute value) zeta potential for the C_{60}FAS indicates its high stability (low tendency for nanoparticle aggregation over time) and suitability for further biological research.

2.2. Biomechanical Analysis

In the process of analysis of the force curves obtained during stimulation of muscle soleus by 5 s pools for 1500 s after chronic intoxication of animals with glyphosate for 30 days, serious disorders in muscle dynamics are visible (Figure 2). The integrated power of muscle contraction during the whole period of stimulation decreased to 41 ± 3% of the control values (Figure 3). A significant reduction in the force response ended in complete muscle rigidity after 1200 s. However, in animals treated with C_{60}FAS, this parameter was 57 ± 2% and 68 ± 4% at doses of C_{60} fullerene 0.5 and 1 mg/kg, respectively. It should be noted that in this case, the muscle responded with a contractile response throughout the stimulation period, not falling below 30% of the limit (Figures 2 and 3). The time of reduction of the force response by 50% and 25% from the initial values increased from 103 ± 11 s and 790 ± 17 s after glyphosate poisoning to 760 ± 8 s and 1213 ± 14 s and 940 ± 21 s and 1820 ± 24 s after therapeutic use of C_{60}FAS at doses of 0.5 and 1 mg/kg, respectively. The maximum and minimum recorded forces of muscle contraction throughout stimulation were 0.81 ± 0.10 N and 0.30 ± 0.05 N after glyphosate poisoning, 1.65 ± 0.20 N and 1.72 ± 0.20 N and 2.67 ± 0.30 N and 2.93 ± 0.30 N after therapeutic use of C_{60}FAS at doses of 0.5 and 1 mg/kg, respectively (Figures 2 and 3).

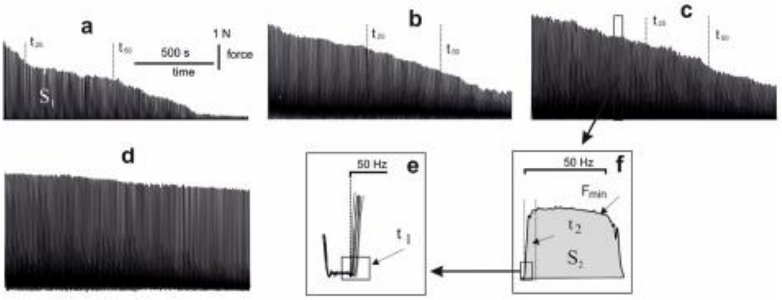

Figure 2. Curves of the generation of the contraction force of muscle soleus rat after chronic intoxication with glyphosate for 30 days: (**a**), (**b**), (**c**) and (**d**)—the curves of muscle contraction for 1500 s with the administration to the animals of glyphosate, glyphosate and C_{60}FAS at doses of 0.5 and 1 mg/kg, respectively, and with the administration to the animals of distilled water (control group); (**e**) mechanograms of single contractions; (**f**) an example of calculating the time of the onset of a muscle response. S_1 is the integrated power of muscle contraction throughout the entire period of stimulation; S_2 is the integrated power in a single contraction; F_{min} is the minimum value of force generation in a single contraction; t_{50} and t_{25} are the time of decreasing the maximum force response to 50% and 25% of the initial amplitude of muscle force; t_1 and t_2 are the time of the onset of the muscle response and the force reaching its maximum value in a single contraction.

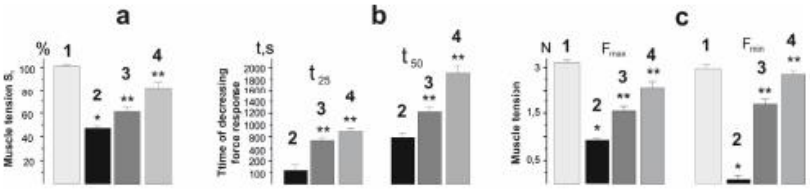

Figure 3. Parameters of contractile activity of muscle soleus rat after chronic intoxication with glyphosate for 30 days: (**a**) integrated power of muscle contraction throughout the entire period of stimulation (S_1), presented as a percentage of control values; (**b**) time of decreasing the force response by 50% (t_{50}) and 25% (t_{25}) from the initial values; (**c**) maximum (F_{max}) and minimum (F_{min}) fixed forces of muscle contraction throughout the entire duration of stimulation. 1—control group (native muscle); 2—the glyphosate group; 3—the glyphosate+C_{60} fullerene (0.5 mg/kg) group; 4—the glyphosate + C_{60} fullerene (1 mg/kg) group; * $p < 0.05$ relative to the control group; ** $p < 0.05$ relative to the glyphosate group.

A decrease in the strength activity of a muscle during glyphosate poisoning can be explained by a violation of energy metabolism. So, in a study [29], the authors found that a high concentration of the herbicide led to a significant decrease in the energy reserve in the muscles, showing an unfavorable sublethal effect on energy metabolism and, consequently, on the dynamic properties of the muscular system in general. The recorded significant positive therapeutic effect of C_{60} fullerene may be associated exclusively with its antioxidant properties, which reduce the degree of damage to cell membranes. To confirm this, we analyzed the biomechanical parameters of single muscle contractions (Figure 4).

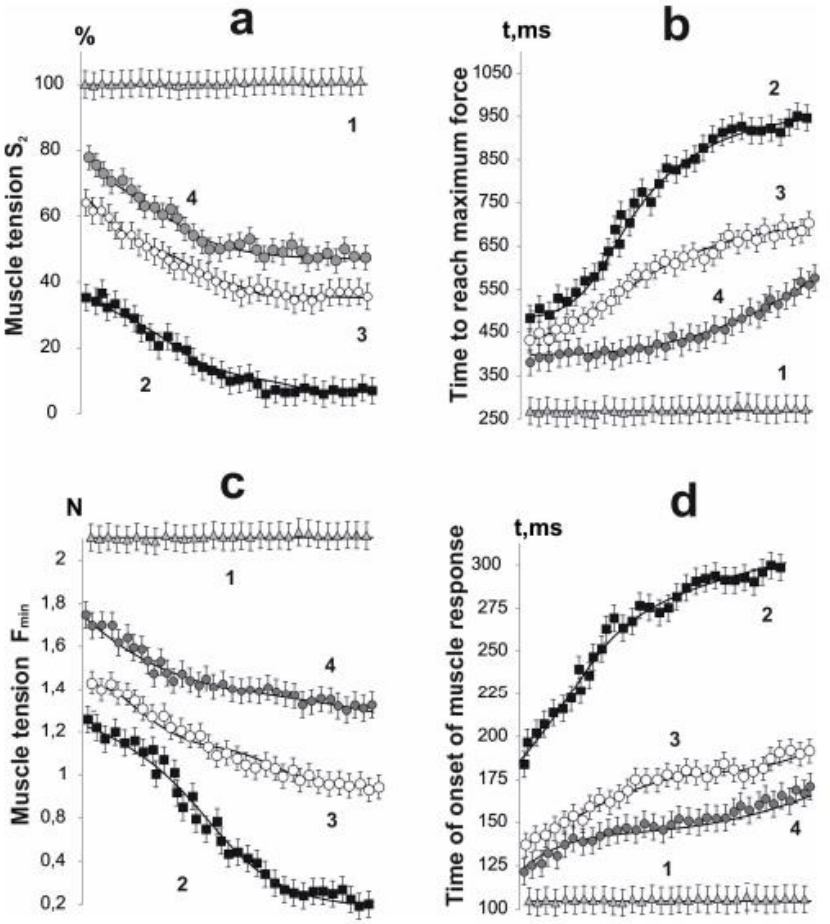

Figure 4. Parameters of single contractions of muscle soleus rat after chronic intoxication with glyphosate for 30 days, caused by 5 s stimulation with a frequency of 50 Hz: (**a**) integrated muscle power (S_2), calculated from the total area of the force curves as a percentage of the control values; (**b**) time to reach the maximum force response; (**c**) minimum (F_{min}) fixed force of muscle contraction; (**d**) time of onset of muscle response to stimulation. 1—control group (native muscle); 2—the glyphosate group; 3—the glyphosate+C_{60} fullerene (0.5 mg/kg) group; 4—the glyphosate+C_{60} fullerene (1 mg/kg) group.

The dynamics of the contractile component is determined by the sensitive mechanisms of interaction of motor neuron pools with actin and myosin myofilaments. The influence of pathological factors on these processes leads either to a complete dysfunction of this process, or to its desynchronization. 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 (CNS). The nature and level of such dysfunctions is directly related to the level of development of pathological processes in both muscle and nervous tissue. The results of studies [30] show that the effect of the glyphosate-based herbicide affects the CNS of rats, possibly altering the neurotransmitter systems that regulate locomotor activity.

The experiment made it possible to trace the therapeutic effect of C_{60}FAS on different regions of the generation of the force response of the rat muscle after chronic intoxication

with glyphosate (Figure 4). A change in the time the force reaches its maximum level is one of the most important parameters of the kinetics of skeletal muscle contraction. This component of muscle dynamics is especially important in controlling hand contraction in humans. Pathological processes occurring in the nervous or muscle tissue lead to its increase, which complicates, and in some cases completely blocks the possibility of accurate positioning of the joint with the damaged muscle [31]. After taking glyphosate for 30 days, this parameter increased significantly. It should be noted that this increase was progressive with growing in the number of contractions: from 470 ± 27 ms with the first contraction to 954 ± 33 ms with the last contraction (in control, 250 ± 11 ms). These values changed significantly after the therapeutic use of CωFAS: with a dose of Cω fullerene of 0.5 mg/kg, this time was 430 ± 22 ms and 650 ± 29 ms, respectively, and with a dose of 1 mg/kg—367 ± 19 ms and 543 ± 24 ms, respectively (Figure 4). Thus, the protective effect of CωFAS was more than 30% in the first and more than 65% in the second cases.

To understand the features of muscle dynamics during the development of a pathological process, it is important to analyze the rate of processing of stimulation pools emanating from the CNS into the mechanical component of contraction and the possibility of modifying the kinetics of contraction under the influence of pathological changes. A change in the time of the onset of muscle response after nerve stimulation is one of the most important parameters of the kinetics of skeletal muscle contraction. Analysis of the data obtained showed a significant increase in this parameter after glyphosate poisoning from 175 ± 22 ms with the first contraction to 298 ± 27 ms with the last compared with the control—102 ± 8 ms. CωFAS therapy at a dose of Cω fullerene 0.5 mg/kg reduced this time to 132 ± 19 ms and 184 ± 17 ms during the first and last muscle contractions, respectively, and with a dose of Cω fullerene 1 mg/kg—to 120 ± 20 ms and 165 ± 14 ms, respectively. It should be noted that significant decrease in this indicator under the action of both doses of Cω fullerene (Figure 4): the protective effect of CωFAS was more than 65% in the first case and more than 75% in the second case.

A change in the level of minimum force of muscle contraction generation is an indicator of significant changes caused by pathological processes in the myocyte. This indicator is not associated with neuropathic damage and its analysis gives an idea of violations of the force generation system within the muscle fiber. When performing fairly simple single-joint movements, this marker is the main indicator of muscle dysfunction, the phenomenological analysis of which makes it possible to establish the presence of causal relationships between the levels of decrease in the biomechanical activity of muscles and the development of the pathological process [32]. With a constant level of the minimum force of more than 2 N in the control, its drop with the use of glyphosate ranged from 1.3 ± 0.1 N to zero. CωFAS therapy increased the level of the minimum force of muscle contraction to (1.4–0.9) ± 0.1 N at a dose of Cω fullerene 0.5 mg/kg and up to (1.7–1.4) ± 0.1 N at a dose of Cω fullerene 1 mg/kg, respectively. In this case, the protective effect of CωFAS was more than 50% in the first case and more than 75% in the second case.

All these changes ultimately lead to a change in the overall strength activity of the muscle, which can be quantified by the value of the integrated power. A change in this parameter can be associated with disorder in both neural component and muscular component of the studied pathology [33]. With chronic use of glyphosate, the integrated power decreased from 41 ± 3% to zero. CωFAS therapy brought this level to 63 ± 3%—47 ± 5% at a dose of Cω fullerene 0.5 mg/kg and 80 ± 6%—54 ± 2% at a dose of Cω fullerene of 1 mg/kg, respectively. The protective effect of CωFAS was more than 50% in the first case and more than 60% in the second case.

2.3. Biochemical Analysis

Analysis of biochemical markers of rat blood, in particular creatinine, creatine phosphokinase (CPK), lactate (LA) and lactate dehydrogenase (LDH), makes it possible to assess the physiological changes occurring in skeletal muscle and the effect of a therapeutic drug on pathological processes in it. Studies have shown that the levels of the selected

markers have a pronounced tendency to increase in the blood of rats intoxicated with glyphosate and decrease during C60FAS therapy (Figure 5).

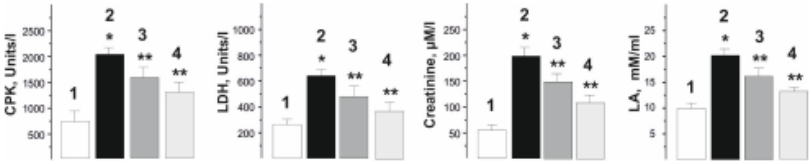

Figure 5. Biochemical parameters of rat blood (CPK, LDH, creatinine and LA) after chronic glyphosate intoxication for 30 days. 1—control group (native muscle); 2—the glyphosate group; 3—the glyphosate+C60 fullerene (0.5 mg/kg) group; 4—the glyphosate+C60 fullerene (1 mg/kg) group; * $p < 0.05$ relative to the control group; ** $p < 0.05$ relative to the glyphosate group.

The change in the concentration of CPK, an enzyme from the energy supply system of musculoskeletal cells, from 756 ± 26 U/L in the norm to 1950 ± 33 U/L after glyphosate intoxication, in our opinion, may be the result of destruction of myocyte walls caused by the influence of the herbicide, with partial release of intramyocytic enzymes into the extracellular space. With the use of C60FAS, the CPK level decreased by 23.2 ± 3% and 31.7 ± 2% at doses of C60 fullerene 0.5 and 1 mg/kg, respectively (Figure 5).

Analysis of changes in the level of LDH made it possible to assess the overall health of the injured muscle. The increase in the LDH level after administration of glyphosate increased from 254 ± 13 U/L (normal) to 659 ± 26 U/L and is evidence of the development of significant dysfunctions of the neuromuscular drug and, as a consequence, the development of fatigue processes. After therapeutic use of C60FAS, the LDH level decreased by 27 ± 3% and 31 ± 2% at doses of C60 fullerene 0.5 and 1 mg/kg, respectively.

The change in creatinine level from 50 ± 2 μM/L in the control to 196 ± 4 μM/L with chronic intake of glyphosate confirms previously obtained data that increased serum creatinine level is an important factor for predicting the severity of glyphosate poisoning [34]. C60FAS therapy led to a significant decrease in its levels to 157 ± 3 μM/L and 112 ± 4 μM/L at doses of C60 fullerene 0.5 and 1 mg/kg, respectively. In our opinion, the decrease in the creatinine fraction in this case is caused by the antioxidant properties of C60 fullerene, its ability to reduce inflammatory reactions and protect the membranes of skeletal muscle cells from nonspecific free radical destruction by efficient absorption of free radicals [35].

Contraction of skeletal muscles leads to the accumulation of LA and H^+ ions and, accordingly, to acidification of the intra- and extracellular media, which reduces the production of ATP and suppresses the activity of Na^+, K^+-ATPase. This leads to a delay in the generation of action potentials and reduces muscle activity. Pathological processes in the myocyte increase this imbalance towards acidification of the medium and, thus, the LA level is an important marker for assessing the degree of muscle activity. Analysis of the LA level showed its increase from 10 ± 1 mM/mL (normal) to 19 ± 2 mM/mL after using glyphosate. The use of C60FAS therapy reduced its level to 16 ± 2 mM/mL and 14 ± 1 mM/mL at doses of C60 fullerene 0.5 and 1 mg/kg, respectively.

Glyphosate is an endocrine disruptor in chronic ingestion, exhibiting high cytotoxicity. The previously obtained results [36] show that it affects survival due to deregulation of the cell cycle and metabolic changes that can alter mitochondrial oxygen consumption, increase free radical levels, damage DNA, cause hypoxia, accumulation of mutations and, ultimately, cell death. It was also shown that after exposure to the herbicide for 8 days at a concentration of 0.95 mg/L, there was an increase in the amount of TBARS in muscle and brain tissues. An increase in reduced glutathione (GSH) level also indicated a compensatory response of the body against toxic conditions. Oxidative stress that arose during the period of exposure to the herbicide was probably caused by increased LPO [30]. Thus, a change in the level of endogenous antioxidants is an important marker that determines the degree of physiological disorders in muscle cells during glyphosate intoxication.

Figure 6 shows the results of measurements of indicators of pro- and antioxidant balance in the blood of experimental rats. The data obtained indicate increased levels of peroxidation and oxidative stress as well as endogenous antioxidants with the use of the herbicide. The increase in these biochemical markers compared to control values was 218 ± 19%, 251 ± 14%, 280 ± 19% and 250 ± 24% for TBARS, hydrogen peroxide (H_2O_2), GSH and catalase (CAT) activity, respectively.

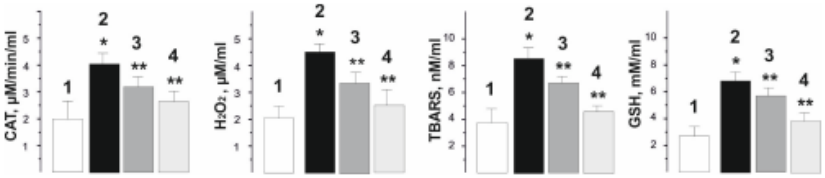

Figure 6. Indicators of pro- and antioxidant balance (CAT, H_2O_2, TBARS and GSH) in the blood of rats after chronic intoxication with glyphosate for 30 days. 1—control group (native muscle); 2—the glyphosate group; 3—the glyphosate+C_{60} fullerene (0.5 mg/kg) group; 4—the glyphosate+C_{60} fullerene (1 mg/kg) group; * $p < 0.05$ relative to the control group; ** $p < 0.05$ relative to the glyphosate group.

The level of these markers decreased significantly after therapeutic use of C_{60}FAS. So, the TBARS level decreased to 170 ± 11% and 120 ± 8% of the control values, H_2O_2—160 ± 14% and 114 ± 11%, GSH—150 ± 12% and 128 ± 9%, CAT activity—140 ± 14% and 119 ± 10% at doses of C_{60} fullerene 0.5 and 1 mg/kg, respectively.

Summarizing, the proposed therapy with the use of low doses of water-soluble C_{60} fullerenes, possessing membranotropic [25,37] and powerful antioxidant properties [38], leads to positive biomechanical and biochemical changes in the character of contractile processes in the skeletal muscles of rats with chronic glyphosate intoxication.

3. Materials and Methods

To obtain C_{60}FAS (maximum concentration 0.15 mg/mL), a method based on the transfer of these carbon molecules from toluene to water followed by sonication was used [27,39]. The prepared C_{60}FAS was stored at a temperature of +4 °C for 12 months.

The AFM (Solver Pro M system, NT-MDT, Moscow, Russia) was performed to determine the size of C_{60} fullerene particles in the prepared aqueous solution. A drop of investigated solution was transferred on the atomic-smooth substrate to deposit layers. Measurements were carried out after complete evaporation of the solvent. For AFM study, a freshly broken surface of mica (SPI supplies, V-1 grade) was used as a substrate. Measurements were carried out in a semicontact (tapping) mode with AFM probes of the RTPESPA150 (Bruker, 6 N/m, 150 kHz) type.

The zeta potential was measured to assess the stability of the prepared C_{60}FAS using the Zetasizer Nano-ZS90 technique (Malvern, Worcestershire, UK).

The experiments were performed on male Wistar rats aged 3 months weighing 170 ± 5 g. The study protocol was approved by the bioethics committee 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 №3446—IV 21.02.2006, Kyiv, on the Protection of Animals from Cruelty during medical and biological research.

In total, 40 rats divided into four groups (10 animals each) were used in the study. Glyphosate was administered daily at a dose of 10 μg/kg of animal weight orally using a metal catheter for 30 days ($n = 10$). The animals of the control group ($n = 10$) were injected with an equivalent volume of distilled water for 30 days. C_{60}FAS was administered at

doses of 0.5 (n = 10) and 1 mg/kg of animal weight (n = 10) immediately after administration of the herbicide for 30 days. Measurements of the studied parameters (see below) in all groups were performed on the 31st day after the start of the experiment.

It should be noted that the use of selected doses of C_{60}FAS are based on previous experimentally established data, which showed a high protective effect of water-soluble C_{60} fullerenes [13,14,19]. Additionally, it should be noted that the doses of C_{60} fullerene used in our experiments are significantly lower than the LD_{50} value, which was 600 mg/kg body weight when administered orally to rats [40] and 721 mg/kg when administered intraperitoneally to mice [25].

Anesthesia of animals was performed by intraperitoneal administration of nembutal (40 mg/kg). Preparation of the experiment included the cannulation (*a. carotis communis sinistra*) for the therapeutic administration of the drug and pressure measurement, tracheotomy and laminectomy at lumbar spinal cord level. Muscle soleus of rat was released from the surrounding tissues. Its tendon was cut across in distal part, which was connected to the force sensors. For modulated stimulation of efferents, the ventral roots were cut at the points of their exit from the spinal cord. Stimulation of efferents was performed by electrical pulses lasting 2 ms, generated by the generator, through platinum electrodes. The control of the external load on the muscle was performed using a system of mechanical stimulators. Perturbation of the load was carried out by a linear electromagnetic motor [41].

The choice of muscle soleus for this study is due to the fact that this muscle contains the maximum number of slow fibers, which is important for accurate and high-quality fixation of fast-acting processes, occurring in the anterior front of the tetanus, in pathology.

To induce muscle contraction, a stimulation signal with a frequency of 50 Hz and a duration of 5 s was used without a relaxation period. The total duration of stimulation was 1500 s. The current strength, at which the muscle began to contract, was considered a threshold, and further stimulation was performed with a current strength of 1.3–1.4 thresholds.

To record the force of skeletal muscle contraction, we used the original strain gauge that consists of force and length sensors, a synchronous pulse generator and a thermal control system [13].

In the process of analyzing the obtained results, the following parameter was used: the integrated power of muscle contraction (calculated area under the force curve), which is an indicator of the overall performance of the muscle with the applied stimulation pools. The development of muscle contractile activity was assessed by calculating the time of the decrease in the force response by 50% and 25% of the initial values during stimulation. We also analyzed the time to reach the maximum value of the muscle contraction force and the delay in the onset of the muscle response.

The level of enzymes content in the blood of experimental animals (creatinine, CPK, LA, LDH, TBARS, H_2O_2, GSH and CAT), as marker of muscle injury [42], was determined using clinical diagnostic equipment—a haemoanalyzer [13].

Statistical processing of results was performed by methods of variation statistics using software Original 9.4. We conducted at least six repetitions for each measurement. Data are expressed as the means ± 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.

4. Conclusions

The obtained results indicate that the therapeutic administration of water-soluble C_{60} fullerenes at a dose of 0.5 mg/kg reduces the degree of pathological changes in rats caused by chronic glyphosate intoxication by 40–45%. Increasing the dose of water-soluble C_{60} fullerenes to 1 mg/kg increases the therapeutic effect by 55–65%, normalizing the studied biomechanical and biochemical parameters. Considering the fact that poisoning with

glyphosate compounds has a lethality of up to 20% and there is currently no antidote to them, and the basis for the treatment of systemic toxicity is deactivation and aggressive supportive therapy [34], the proposed C_{60} fullerene therapy of this type of intoxication opens up new prospects for clinical trials.

Author Contributions: Biomechanical analysis, D.N., O.A. and V.S.; biochemical analysis, S.P., O.V. and K.B.; sample preparation, U.R. and P.S.; characterization of the samples, S.K. and Y.P.; coordination the research work, analysis of the data and preparing of the manuscript, Y.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study protocol was approved by the bioethics committee 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 №3446—IV 21.02.2006, Kyiv, on the Protection of Animals from Cruelty during medical and biological research.

Informed Consent Statement: Not applicable.

Data Availability Statement Not applicable.

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Conflicts of Interest: Authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Post-traumatic recovery of *muscle soleus* in rats is improved via synergistic effect of C_{60} fullerene and TRPM8 agonist menthol

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Abstract
Functional biomechanical parameters of *muscle soleus* contraction in rats as well as selected blood biochemical parameters were studied during the first 3 days of post-traumatic syndrome progression caused by the destruction of muscle cells by compression. Single administration of the antioxidant C_{60} fullerene and the selective agonist of TRPM8 channels menthol were used as therapeutic agents. Injection of C_{60} fullerene at a concentration of 1 mg/kg into the damaged muscle improved its contractile function by 25–28%. The use of combined injections of C_{60} fullerene and menthol (at the concentration 1 mg/kg) improved this index by additional 27–39% and simultaneously stabilized the decrease in muscle strength observed throughout the experiment. A tendency towards a decrease in the indexes of the above described biochemical parameters by 10–15% were found with the therapeutic administration of C_{60} fullerene. With combined injections of C_{60} fullerene and menthol, the above described biochemical parameters decreased by an additional 17–24%. The synergism between the action of menthol and C_{60} fullerene on the post-traumatic recovery of skeletal muscle function opens up new perspectives for the clinical application of this combination therapy.

Keywords Rat *muscle soleus* · Muscle injury · C_{60} fullerene · Menthol · TRPM8 channel · Biomechanical and biochemical parameters

Abbreviations

C_{60}	C_{60} fullerenes
C_{60}FAS	C_{60} fullerene aqueous solution
CAT	Catalase
CS	Crush syndrome
CPK	Creatine phosphokinase
H_2O_2	Hydrogen peroxide
GSH	Reduced glutathione
LA	Lactate
LDH	Lactate dehydrogenase
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances
TRP channels	Transient Receptor Potential channels

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Introduction

Despite the high medical and social significance of skeletal muscle injuries, there are quite a few clinical options for their treatment. Timely therapy, especially at the early stages of such pathology, can optimize the regeneration and healing of the damaged skeletal muscles and help prevent the risk of some serious post-traumatic complications while accelerating full muscle recovery. The reaction of the skeletal muscle tissue to the action of the harmful factor(s) has a distinct phase character and is manifested by alteration (stage of inflammation), exudation (release of fluid and blood cells from blood vessels into tissues and organs) and proliferation (recovery phase). In parallel with muscle tissue disorders there is a complex of vascular changes in the form of short-term spasm, arterial and venous hyperaemia. The occurrence of an inflammatory reaction is a consequence of the appearance of a large number of free radical agents in damaged tissues, which trigger a cascade of pathological processes resulting primarily in altered integrity of cell membranes. It is well established that free radicals, particularly superoxide and hydroxyl radicals, are the main factors in the process of muscle tissue damage. Thus, they initiate lipid peroxidation, cause direct inhibition of mitochondrial enzymes of the respiratory chain and ATPase activity, inactivation of glyceraldehyde-3-phosphate dehydrogenase and membrane sodium channels (Cuzzocrea et al. 2001). Biocompatible and bioavailable carbon nanoparticles C_{60} fullerenes (C_{60}) (Halenova et al. 2020; Prylutska et al. 2007) can act as powerful scavengers of free radicals (Eswaran et al. 2018; Gonchar et al. 2018) induced by ischemia–reperfusion injury (Amani et al. 2017; Matvienko et al. 2017). However, our earlier studies (Nozdrenko et al. 2017) were related to relatively mild muscle pathologies. Muscle injury with rupture of muscle tissue is a severe pathology complicated by pronounced pain symptoms. Therefore, the use of C_{60} therapy, in our opinion, is not a sufficiently comprehensive approach for an adequate model of the forthcoming study.

The ability to perceive temperature stimuli provides a basis for the formation of adaptive responses aimed at the active elimination of the pathological process. In recent decades, several members of the superfamily of Transient Receptor Potential (TRP) channels have been identified as specific thermoreceptors. Accumulating evidence indicates their potential participation in a number of physiological processes that contribute to the alleviation of pathological conditions (Nilius et al. 2007; Zholos et al. 2011). Thus, it has been established that menthol increases muscle endurance during exercise, reducing levels of lactic acid and triglycerides in the blood by activating TRPM8 channels, and thus improving energy metabolism of skeletal muscles (Chen 2018 et al.). Long-term studies have shown that therapeutic use of a menthol-containing drug significantly reduces time for return to sports activity in athletes with injuries of varying severity (Isbary et al. 1983). Positive effect of menthol on the rate of the strength recovery of muscles contraction of the athlete's lower body after physical exertion was established (Gillis et al. 2020). In the context of muscle trauma, it is especially relevant that TRPM8 agonist can normalize blood circulation by exerting dual effect on vascular tone – vasorelaxation of constricted blood vessels and vasoconstriction of blood vessels at rest (Johnson et al. 2009).

Based on these observations, the aim of this work was to investigate the possibility of a synergistic effect of the therapeutic action of C_{60} as an antioxidant and menthol as an activator of TRPM8 channels, on post-traumatic restoration of the functioning of the *muscle soleus* in rats.

Materials and methods

To obtain the C_{60} fullerene aqueous solution (C_{60}FAS) we used a method based on the transfer of these carbon nanostructures from toluene to water, followed by sonication (Ritter et al. 2015; Scharff et al. 2004). The obtained C_{60}FAS is a typical colloid solution that contains both single C_{60} (~0.72 nm) and their nanoaggregates with a size of 1.2–100 nm (Prilutski et al. 1998). C_{60}FAS was stable at 4 ^{0}C for 18 months.

50 male Wistar rats aged 3 months weighing 170 ± 5 g were used in the experiments. The study protocol was approved by the Bioethics Commission of ESC "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv in accordance with the European Convention for the Protection of Vertebrates animals used for experimental and other scientific purposes" and norms of biomedical ethics in accordance with the Law of Ukraine №3446—IV 21.02.2006, Kyiv, "On protection of animals from cruel treatment" during medical and biological research.

The animals were anaesthetized by intraperitoneal administration of nembutal (40 mg/kg). Muscle injury was induced by squeezing the muscle for 1 min with a clamp at a pressure of 3.5 kg per cm^3 (Souza et al. 2013). The applied crush syndrome (CS) led to the systemic manifestation of pathological changes due to destruction of muscle cells, particularly, the release of muscle cell components (creatine kinase, lactic acid, myoglobin) into the extracellular environment, which served as a marker of muscle injury.

Preparation of the experiment included cannulation (*a. carotis communis sinistra*) for the pharmacological drugs administration and measurement of blood pressure, tracheotomy and laminectomy at the level of the lumbar spinal cord.

The rat *muscle soleus* was separated from the surrounding tissues. In the distal part, its tendon part was cut transversely. For modulated stimulation of efferents in segments L7-S1, the ventral roots were cut at the points of their exit from the spinal cord. Stimulation of efferents was performed by electrical pulses lasting 2 ms, generated by a pulse generator through platinum electrodes. Control of the external load on the muscle was performed using a system of mechanical stimulators. Perturbation of the load was carried out by a linear electromagnetic motor (Nozdrenko et al. 2018).

To induce muscle contraction, a three-component stimulation signal with a frequency of 1 Hz was used, each lasting 10 min with a relaxation period between pools of 100 s. The strength of the current at which the muscle began to contract was considered as threshold, further stimulation was performed at strength of 1.3–1.4 threshold.

C_{60}FAS and menthol were administered sequentially intramuscularly at a concentration of 1 mg/kg body weight immediately after initiation of muscle injury. It is important to note, that accordingly our previous study, the maximum tolerated dose of C_{60}FAS is 721 mg/kg for *i.p.* administration to mice (Prylutska et al. 2019).

To record the force of skeletal muscle contraction, we used a custom made strain gauge, which included force and length sensors, a synchronous pulse generator and a thermal control system (Nozdrenko et al. 2017).

Integrated muscle power, which is an indicator of the general performance of the muscle during the application of stimulation pools, was calculated as the area under the force curve. The analysis of this parameter made it possible to assess the mechanisms of the formation of muscular activity at the equilibrium state in the "force—external load" system, which is a physiological analogue of the performance of the muscular system as a whole. Mechanograms were analyzed on days 1, 2, and 3 after muscle injury.

The development of muscle contractile activity was assessed by the method of calculating time intervals during which 50% and 25% of the initial levels of force responses were reached during muscle stimulation.

The level of biochemical changes in the blood of experimental animals (creatinine, creatine phosphokinase (CPK), lactate (LA), lactate dehydrogenase (LDH), thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H_2O_2), reduced glutathione (GSH) and catalase (CAT)), as markers of muscle injury, was determined using clinical diagnostic equipment—a haemoanalyzer (Nozdrenko et al. 2017).

Statistical analysis

Statistical assessment of the results was performed by methods of variation statistics using OriginPro 2020 (v. 9.7) (OriginLab, Northampton, MA, USA). At least six replicates were performed for each measurement. Data are expressed as the means ± SEM for each group. The differences among experimental groups were detected by one-way ANOVA followed by Bonferroni's multiple comparison test. Differences at $p < 0.05$ were considered significant.

Results and discussion

Biomechanics of injured muscle contractions

Applied crush syndrome (CS), as a factor of muscle injury, caused a significant decrease in the force response of the muscle with progressive temporal symptoms (Fig. 1). Thus, force responses to a stimulus showed sharp decrease in force activity of the muscle already in the first seconds of stimulation with progressive decrease in the maximum force to 21 ± 1% of initial values on the first pool of stimulation, 17 ± 1% and 9 ± 1% during the second and third pools, respectively ($n = 10$). The decrease in integrated muscle contraction power was 53 ± 2%, 42 ± 1%, and 23 ± 1% ($n = 10$) in the first, second, and third stimulation pools, respectively (Fig. 1). The time to reach 50% and 25% of the initial level of force response was 156 ± 5 and 401 ± 2 s during the first stimulation pool, 143 ± 3 and 376 ± 3 s during the second and 122 ± 2 and 311 ± 2 s ($n = 10$) during the third stimulation pool. Thus, there are progressive fatigue processes of the injured muscle and insufficient relaxation time for its adequate functioning. It is important to note that the intact muscle under these conditions does not change its strength characteristics during stimulation for several hours (Nozdrenko et al. 2017).

The use of C_{60} injections increased the muscle strength response on average by 9–12% during the first stimulation pool. During the second and third stimulation pools, the muscle strength response did not significantly increase and averaged 5–7% of the control values (Fig. 1, injury + C_{60}). Menthol (M) injections alone did not result in any significant changes in muscle dynamics (Fig. 1, injury + M)).

The use of combined injections of C_{60} and menthol showed a significant increase in muscle strength during all three stimulation pools. The integrated power increased by 58 ± 1%, 42 ± 2% and 36 ± 2% during the first, second and third pool, respectively ($n = 10$). The time to reach 50% and 25% of the initial level of force response increased by almost 50% in each of the three stimulation pools (Fig. 1, injury + C_{60} + M).). It is also important to note the absence of a sharp decrease in the maximum force of contraction during the first seconds of stimulation: the decrease in force occurred smoothly and monotonously during all three stimulation pools.

Next we analyzed the changes in muscle dynamics on days 1, 2, and 3 after muscle injury. The decrease in the

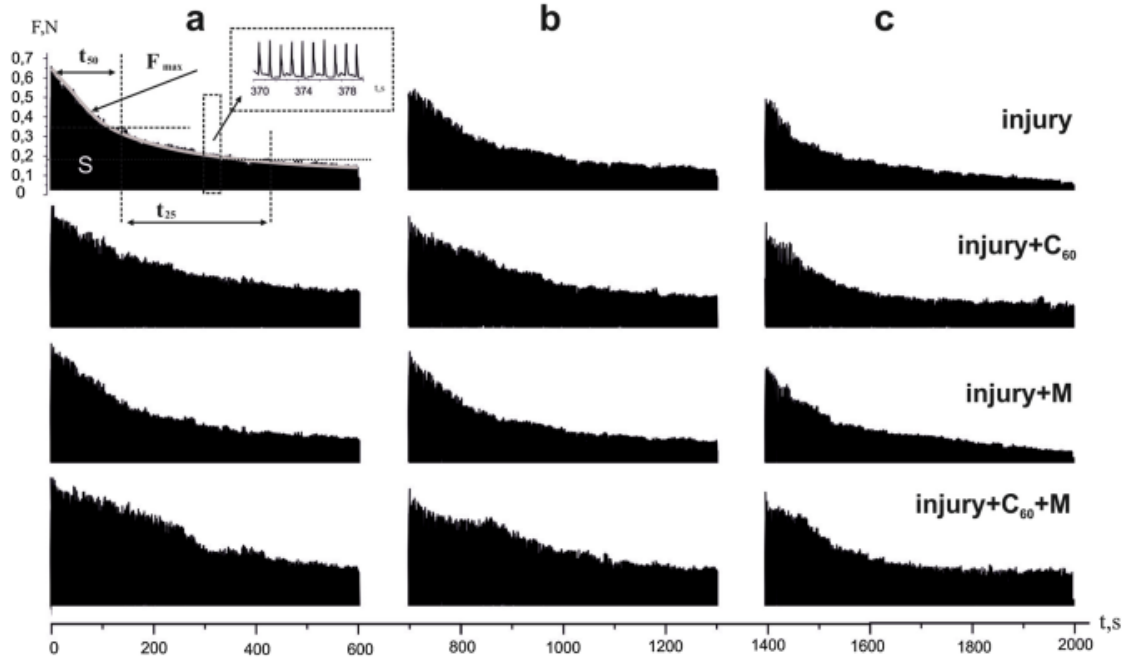

Fig. 1 Curves of the generation of the contraction force of the *muscle soleus* 5 h after the initiation of traumatic injury caused by a three-component stimulation pulses applied at 1 Hz, 600 s duration each with a relaxation period between pools of 100 s: a, b, c—three consecutive pools of stimulation; S is the integrated power of muscle contraction; F_{max}—curve of the maximum force response of the muscle (N); t_{50} and t_{25}—time of the decrease in the maximum strength response to 50% and 25% of the initial amplitude of force of muscle contraction, respectively; "injury + C_{60}" and "injury + C_{60} + M" indicate treatments by injections of C_{60} and C_{60} with menthol (M), respectively

integrated power of muscle contraction was by 46 ± 1%, 31 ± 1% and 15 ± 1% (n = 10) of the control values on days 1, 2, and 3 after muscle injury, respectively. The time of the reduction in the maximum power indicators by 50% was 100 ± 2, 78 ± 1 and 54 ± 2 s (n = 10), and by 25%—121 ± 1, 107 ± 1 and 78 ± 2 s (n = 10) by 1, 2 and 3 days after the injury, respectively (Fig. 2). A temporal analysis of the healing of *muscle soleus* injury showed that complete muscle regeneration occured 3–5 days after the injury (Hurme et al. 1991).

The use of C_{60} injection significantly improved the dynamics of the contractile process of the damaged muscle. Thus, the decrease in the integrated power was by 63 ± 2%, 58 ± 2% and 42 ± 2% (n = 10) of the maximum values of the force on days 1, 2, and 3 after the injury, which was 27–30% less than for the injured muscles without treatment. The time to reach the maximum power indicators of 50% of the level from the initial values was 256 ± 11, 321 ± 9 and 211 ± 5 s (n = 10), and 25% of the initial level—325 ± 9, 301 ± 7 and 276 ± 6 s (n = 10) on days 1, 2 and 3 after the injury, respectively, which was by 30–32% more compared to the injured muscle in control (Fig. 2). Thus, these data indicate a significant positive dynamics of the therapeutic administration of C_{60}FAS. However, it should be noted that this therapy alone did not lead to significant biomechanical changes regarding the nature of the contractile processes. We suppose that inactivation of the produced free radicals by C_{60} reduces the level of injury severity in the muscle by 25–30%, which, although it is a positive aspect of this therapy, does not ensure a significant progress in the process of complete recovery of muscle function.

Menthol injections into the injured muscle did not show much positive therapeutic results in improving its dynamic response (data not shown). However, combined treatment with menthol and C_{60} resulted in the decrease of the integrated power by 79 ± 2%, 63 ± 2% and 49 ± 2% (n = 10) of the maximum response on days 1, 2 and 3 after the injury, which was by 40–45% less than for the damaged muscle in control and by 17% less compared to the C_{60} therapy alone. The time to reach the maximum of force indicators by 50% of the initial values was 325 ± 3, 321 ± 4 and 300 ± 3 s (n = 10), and by 25%—300 ± 3, 296 ± 4 and 290 ± 4 s (n = 10) on days 1, 2 and 3 after the injury, respectively (Fig. 2), which was by 42–45% less relative to the damaged muscle in control

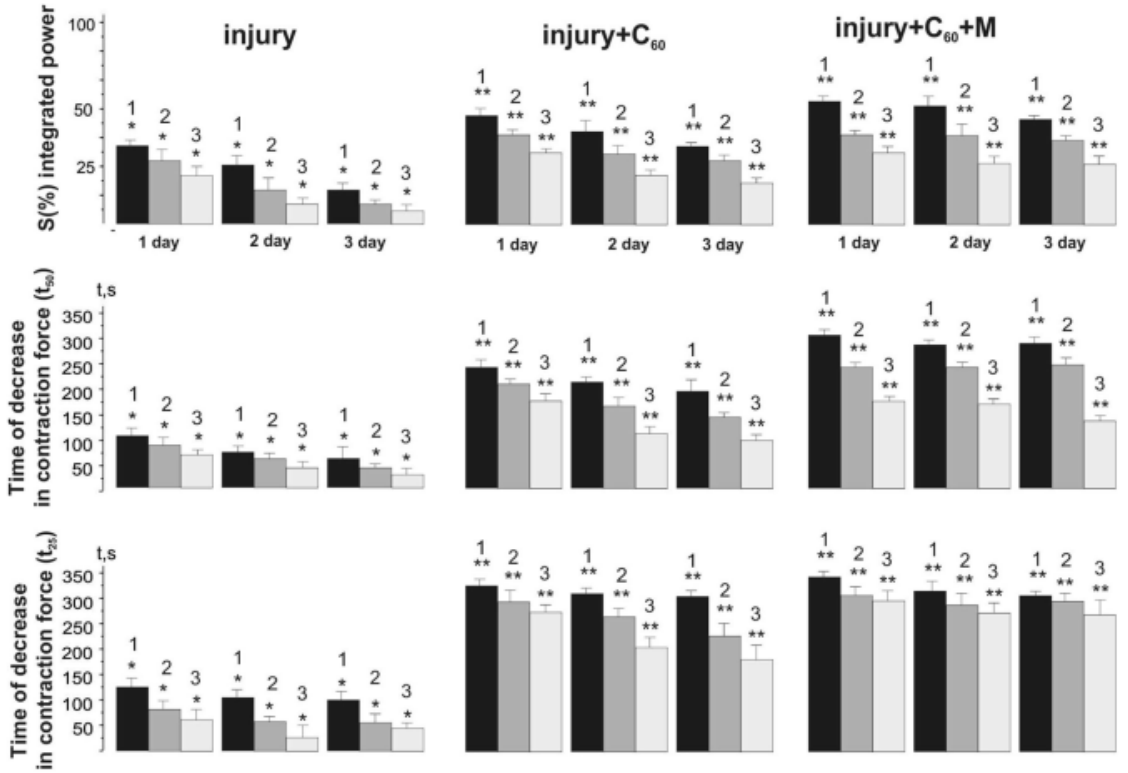

Fig. 2 Biomechanical parameters of muscle fatigue of posttraumatic *muscle soleus* after using therapeutic agents: S—integrated power of muscle contractions; t_{50} and t_{25}—the time to reach 50% and 25% of the initial muscle force of contraction, respectively; "injury + C_{60}" and "injury + C_{60} + M" indicate treatments by injections of C_{60} and C_{60} with menthol (M), respectively; 1, 2, 3—1, 2 and 3 sequential stimulation pool; a, b, c—power responses on days 1, 2 and 3 after muscle injury; $*p < 0.05$; $**p < 0.05$ relative to injury group

and by 19–21% less relative to the C_{60} therapy alone. It is important to note the significant difference in curves of force response after using C_{60} with menthol. A rapid drop in the maximum force response during the first 300 s (half of the muscle stimulation time) was up to 40% of the initial values. In the case of the C_{60} therapy, a gradual decrease of the maximum force of contraction is observed throughout the entire period of stimulation. A possible reason for this may be both a greater number of metabolic components in muscle fibers and stabilization of acidity in the intracellular space. It should also be noted that the use of the combined therapy did not eliminate the developing of fatigue processes in the muscle: the integrated muscle power decreased with each subsequent pool of stimulation (Fig. 2).

Next we analyzed the ability of the injured muscle to maintain maximum force during a 6 s period of muscle stimulation at 50 Hz. Figure 3 shows the curves of ten consecutive force responses of the injured *muscle soleus* 5 h after initiation of the injury. Decrease in the force of contraction during the first five consecutive contractions was replaced by almost complete muscle rigidity during the last stimulation pools. A rapid decrease in the force of contraction during the first stimulation and fluctuating force responses at the last stages of contraction are also notable.

Injections of C_{60} eliminated both the abrupt decrease in the contraction force and the fluctuation component of the contractile process (Figs. 3, 4). However, the integrated power of muscle contraction continued to decrease throughout the duration of stimulation with a slight increase of $12 \pm 3\%$ from the values of the injured untreated muscle. Menthol injections alone did not improve muscle dynamics studied with this protocol (data not shown). However, using combination of C_{60} and menthol resulted in an increase of the integrated power was by $29 \pm 2\%$ of the values of the injured muscle (Fig. 4). Reduction in the maximum force response was evident during each of ten consecutive contractions. C_{60} and menthol treatment increased this parameter during the first stages of contraction only. The decrease of time required for force to reach its maximum level was particularly notable after the combined therapy: from 1.3 s for

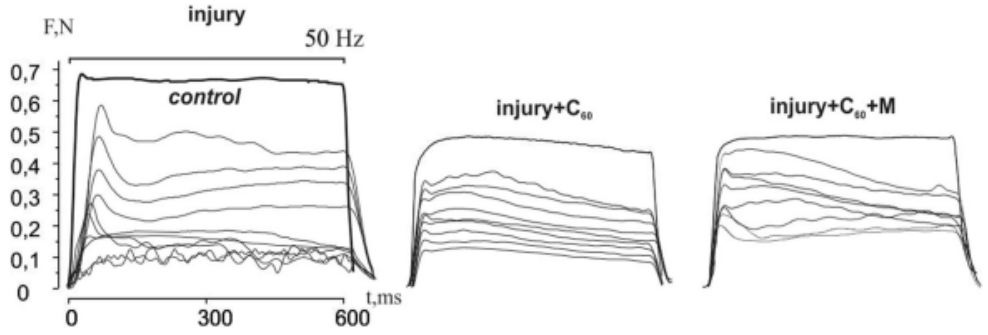

Fig. 3 Curves of 10 consecutive force responses of the injured *muscle soleus* to a stimulation signal with a frequency of 50 Hz of 6 s duration (without a relaxation period) in control (left panel) and after using the therapeutic agents 5 h after the initiation of muscle injury: "injury + C_{60}" and "injury + C_{60} + M" indicate treatments by injections of C_{60} and C_{60} with menthol (M), respectively

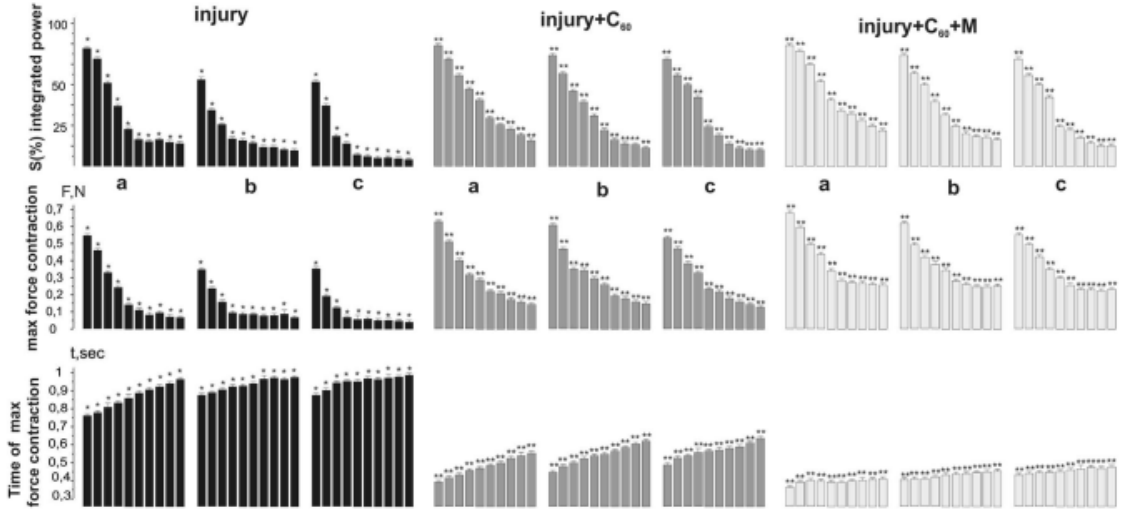

Fig. 4 Biomechanical parameters of 10 consecutive force responses of injured *muscle soleus* to stimulation at 50 Hz for a 6 s period (without a relaxation period) in control (traces marked "injury") and after using therapeutic agents: "injury + C_{60}" and "injury + C_{60} + M" indicate treatments by injections of C_{60} and C_{60} with menthol (M), respectively; a, b, c—curves of maximum strength responses on days 1, 2, and 3 after muscle injury, respectively; $*p < 0.05$; $**p < 0.05$ relative to the injury group

the injured muscle (control) to 0.5 s after the injection of C_{60} and 0.3 s for its combined injection with menthol (Fig. 4).

Analysis of these parameters on days 1, 2 and 3 after muscle injury showed a significant effect of the therapy on each of the studied biomechanical markers (Fig. 4). In the injured muscle, a decrease in the value of the mechanical response of the muscle to the stimulation was observed with an increase in the time after the injury. This is due to the progressive development of inflammatory processes after the initiation of the injury. After using of both C_{60} and C_{60} with menthol the increase of integrated power was by 55, 57, and 59% on days 1, 2 and 3 after the injury, respectively. The increase of maximum force response was by 42 ± 2%, 46 ± 3%, and 158 ± 3% ($n = 10$) on days 1, 2, and 3, respectively, after injection of C_{60} and by 62 ± 1%, 66 ± 2% and 72 ± 3% on days 1, 2 and 3, respectively ($n = 10$) after injections of C_{60} with menthol.

Blood biochemical indicators of rats with injured muscle

Analysis of the selected biochemical blood markers indicative of the quality of skeletal muscle functioning, in particular changes in the levels of creatinine, creatine

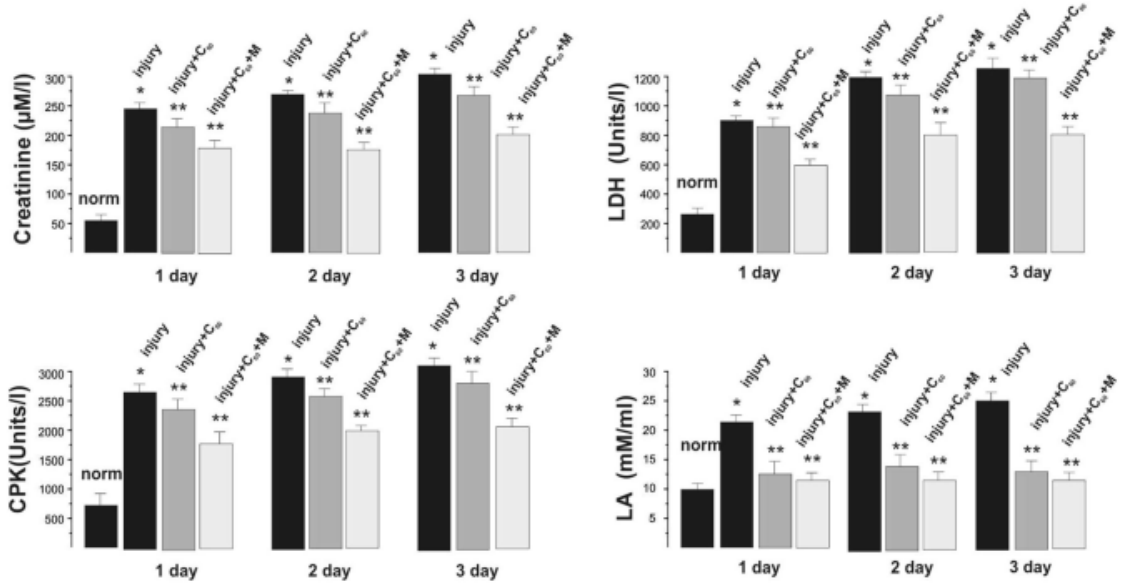

Fig. 5 Changes in the levels of creatinine, lactate dehydrogenase (LDH), creatine phosphokinase (CPK) and lactate (LA) in the blood of rats after muscle injury: "injury + C_{60}" and "injury + C_{60} + M" indicate treatments by injections of C_{60} and C_{60} with menthol (M), respectively; *$p < 0.05$; ** $p < 0.05$ relative to the injury group

phosphokinase (CPK), lactate (LA), and lactate dehydrogenase (LDH) in the blood of rats (Fig. 5), provides opportunities to assess the physiological changes in muscle and the therapeutic effect of the applied drugs on pathological processes. Previous studies have shown that all these markers have a pronounced tendency for elevation with increase in time after initiation of injury that indicates the super-intense for muscular system level of physiological work that is followed by the development of muscle fatigue.

One of the known markers of muscle fatigue is a change in the concentration of CPK, an enzyme involved in the energy supply, which catalyzes the transfer of a phosphate group from ATP to creatine with the formation of a high-energy compound creatine phosphate. After intensive functioning or mechanical damage of the muscles, the release of the enzyme from the cells and increase in CPK activity in the blood are observed. Increase in CPK blood fraction (Fig. 5) from 500 Units/l in control to 2380, 2422, and 2943 Units/l on days 1, 2, and 3 after the injury, respectively, is the result of destruction of myocyte walls caused by muscle injury (Gibala et al. 1995) with partial release of intramyocytic enzymes into the extracellular space. After injection of C_{60}FAS, the CPK level decreased by 9.3, 9.8, and 10.4% ($p < 0.05$) on days 1, 2, and 3, respectively. Combined administration of C_{60}FAS and menthol decreased the CPK level by 16.4, 16.8 and 17.5% ($p < 0.05$), which is evidence of the direct synergistic action of these agents. It is important to note that the use of menthol injections alone did not produce significant differences in any of the biochemical markers compared to the injured muscle.

Changes in LDH, the enzyme that catalyzes oxidation of lactic acid (the end product of glucose metabolism in cells during prolonged physical exertion), provide the means to assess the general state of functionality of the damaged muscle after its prolonged activity. Increase in LDH level from 200 Units/l (normal) to 860, 1180, and 1198 Units/l on days 1, 2, and 3, respectively after muscle injury (Fig. 5) is evidence of the development of significant dysfunction of the neuro-muscular system and, as consequence, the development of fatigue processes. After the injection of C_{60}FAS, LDH level decreased by 4.7, 5.2 and 5.1% ($p < 0.05$) on days 1, 2 and 3 after the injury, respectively. Combined administration of C_{60}FAS and menthol decreased the level of the enzyme by 29.7, 19.5 and 18.3% ($p < 0.05$), which is also significantly higher than with C_{60} therapy alone.

Changes in the level of creatinine, a product formed in muscles during the destruction of intramuscular structures, allowed us to assess the level of damage to myocytes. Figure 5 shows that this index increased from 50 μM/l in control to 240, 2562, and 297 μM/l on days 1, 2, and 3 after the injury, respectively. Injections of C_{60}, as in the analysis of its effect on the above described markers, did not show pronounced changes in creatinine content: creatinine level decreased by 5.3, 5.1 and 4.8% ($p < 0.05$) on days 1, 2, and

3 after the injury, respectively. However, combined administration of C_{60}FAS and menthol caused a marked decrease of creatinine level by 26.2, 27.4 and 26.7% ($p < 0.05$) that is significantly more than with C_{60} therapy alone. It is likely that a decrease in the creatinine fraction after combined therapy by C_{60}FAS and menthol is caused by the protective effect of menthol at the early stages of pathological process development by reducing inflammatory reactions, which then made it possible for C_{60} to show its antioxidant properties and protect the membranes of skeletal myocytes from nonspecific free radical damage by neutralisation of free radicals.

During the development of inflammatory reactions cascade after injury significant depletion of cellular energy substances, especially ATP, occurs, this leads to a disruption of homeostasis and a loss of ion gradients across the cell membranes. This, in turn, results in the accumulation of LA and H^+ ions, and, accordingly, acidification of the intra- and extracellular media (Hagberg et al. 1985). A decrease in ATP production suppresses the activity of the Na^+, K^+-ATPase, which leads to increase in the concentration of intracellular Na^+ and, as a consequence, intracellular Ca^{2+} (Ivanics et al. 2000). The increased content of K^+ ions causes a delay in the generation of the action potential and, accordingly, its propagation along the T-tubules (Jones 1996). Thus, ionic changes impair the muscle's ability to respond to electrical impulses, and lead to a decrease in muscle strength. Therefore, LA is an important marker for assessing the degree of performance of the injured muscle. Analysis of the LA level showed its increase after injury from 10 mM/ml in control to 23, 24, and 26 mM/ml on days 1, 2, and 3, respectively. The use of C_{60} therapy reduced its concentration by 48.5, 46.2 and 47.7% ($p < 0.05$) on days 1, 2, and 3, respectively. However, combined therapy using administration of both C_{60}FAS and menthol practically did not reduce the LA level: the decrease in its level was no more than 3% of the level with C_{60} therapy alone. Thus, under the conditions used for muscle injury development C_{60} is able to significantly reduce the LA level in the active muscle without additional activation of TRPM8 channels.

With the development of muscle pathology, a change in the level of antioxidants is an essential criterion that determines the level of physiological disbalance. The results of tests show the level of accumulation of the secondary products of lipid peroxidation in the blood of rats after induction of muscle injury (Fig. 6). The data obtained indicate an increased level of peroxidation and oxidative stress TBARS (thiobarbituric acid reactive substances) and H_2O_2 (hydrogen peroxide) after stimulation of the injured muscle. These markers increased on the second and third days after the initiation of injury and compared to the intact muscle were 235%, 308% and 423% ($p < 0.05$) for TBARS and 451%,

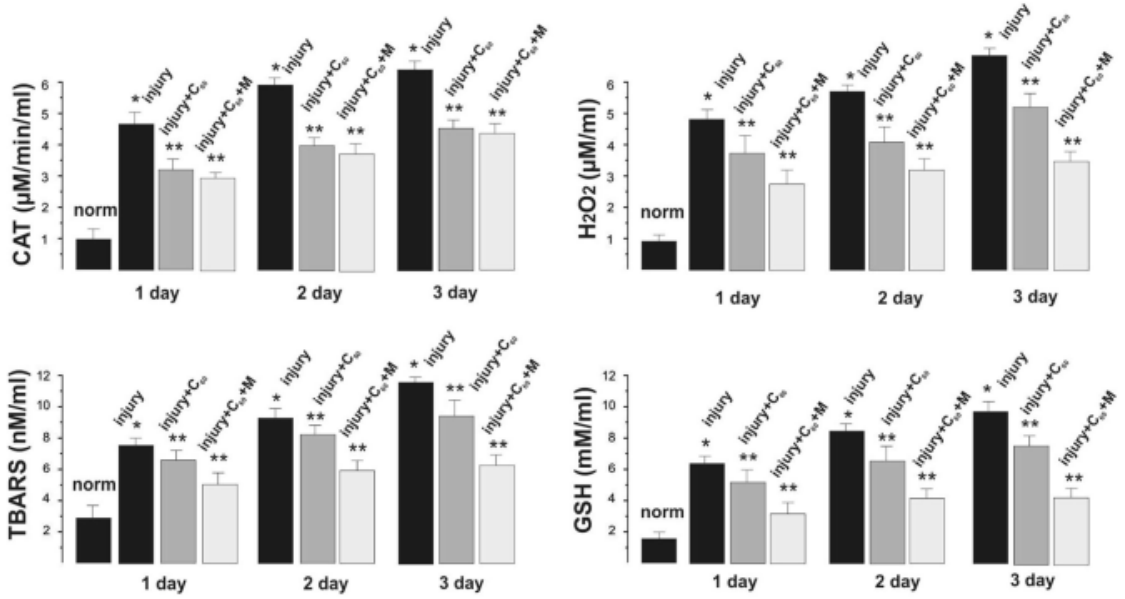

Fig. 6 Indicators of pro- and antioxidant balance in the blood of rats after induction of muscle injury (catalase (CAT) activity, hydrogen peroxide (H_2O_2), thiobarbituric acid reactive substances (TBARS), and reduced glutathione (GSH) concentrations): "injury + C_{60}" and "injury + C_{60} + M" indicate treatments by injections of C_{60} and C_{60} with menthol (M), respectively; *$p < 0.05$; **$p < 0.05$ relative to injury group

522% and 617% ($p < 0.05$) for H_2O_2 on days 1, 2, and 3, respectively. After administration of C_{60}FAS, TBARS concentration slightly decreased compared to the damaged muscle without therapy: 202%, 281%, and 367% ($p < 0.05$) on days 1, 2, and 3, respectively. Thus, the therapeutic effect of C_{60}FAS for this marker was no more than 11%. The decrease in the level of H_2O_2 after administration of C_{60}FAS was no more than 14% (Fig. 6).

The therapeutic effect of C_{60}FAS and menthol combined treatment on TBARS was 31%, 43% and 57% ($p < 0.05$), and on H_2O_2—47%, 49% and 51% ($p < 0.05$) on 1, 2 and 3 days, respectively, which was more than twofold higher than the therapeutic effect of C_{60}FAS alone. It should be noted that the most pronounced therapeutic effect was observed on the 3rd day after muscle injury. Analysis of the levels of the endogenous antioxidants showed a significant increase in GSH (reduced glutathione) levels—301%, 390% and 421% ($p < 0.05$) and activity of antiperoxide enzyme CAT (catalase)—471%, 527% and 578% ($p < 0.05$) on days 1, 2 and 3, respectively. GSH activity slightly decreased by 9%, 11% and 12% ($p < 0.05$), respectively, after administration of C_{60}FAS. The decrease in CAT indicators turned out to be more effective and was 14%, 18% and 19% ($p < 0.05$) on days 1, 2 and 3, respectively. GSH level decreased by almost 50% more than with C_{60} therapy after injections of C_{60}FAS and menthol: the therapeutic effect was 29%, 36% and 41% ($p < 0.05$) on days 1, 2 and 3, respectively. At the same time, the level of CAT remained practically unchanged compared to C_{60} therapy—the difference was no more than 2–3% (Fig. 6).

Significant differences in the severity of injury and the muscle group affected, as well as the nonspecificity of symptoms, complicate research aimed at identification of a suitable treatment for muscle injury. Therefore, it is important to understand the cellular processes inherent to this type of skeletal muscle injury and involved in their healing. The most important of these processes is inflammation as a consistent and sustained systemic response. The inflammatory response depends on two factors, specifically the degree of physical injury and the degree of muscle vascularization during injury. However, long-term anti-inflammatory treatment is not necessarily effective in accelerating healing, as indicated by various (Hurme et al. 1991). Due to a variety of ethical factors, studies of the inflammatory response during injury in humans are limited, but experimental animal models provide sufficient information to study muscle damage and regeneration. However, the methods currently used to induce mechanical damage vary considerably in terms of invasiveness, instruments used to induce injury, the muscle group selected for injury and their contractile status, and the effect on immune or cytokine responses. This complicates the interpretation of the results of such studies.

The early recovery phase of mechanical muscle injury is characterized by overlapping of inflammation process and development of secondary injury. Although neutrophil infiltration has been proposed as one of the reasons for the enhancement of inflammatory process, there is no clear evidence to support this statement. The main role in the initiation of inflammatory reactions is played by cascading, progressive increase in free radical components. Pathological inflammatory cascading processes that occur immediately after muscle injury are the source of free oxygen radicals and contribute to the intensification of lipid peroxidation processes (Davies et al. 1982). The presence of such metabolic products interferes with the adequate performance of muscle work and significantly increases the duration of their recovery period. A decrease in the concentration of these oxygen metabolites upon therapeutic administration of C_{60} should significantly improve the execution of motor commands of the central nervous system by the muscular system and contribute to a decrease in the level of pathological changes. In our opinion, the ability of C_{60} to effectively neutralize free radicals (Ferreira et al. 2018; Vereshchaka et al. 2018), is the main reason for the positive therapeutic results of the treatments described in this study.

Muscle tissue damage, as well as intense exercises, induce oxidative stress in skeletal muscle and therefore can alter the pro-antioxidant balance. Despite numerous studies have been done in this area, connections between free radicals, antioxidant enzymes, exercises, and skeletal muscle membrane injury remain controversial (Clanton et al. 1999; Ji 1995). These discrepancies may be related to differences in levels of tissue injuries, intensity and duration of muscle work, and muscle fiber type. Each type of muscle fibers has different metabolic characteristics and oxidative potential, as well as the ability to provide antioxidant protection (Ji et al. 1999). However, it remains indisputable that the presence of such metabolic products compromises the adequate performance of muscle work and significantly increases the duration of their recovery period.

It should be noted that during the development of inflammatory response of the muscle to traumatic injury in the primary alteration zone, the metabolic rate is reduced due to cellular dysfunctions, and in the secondary alteration zone it is increased due to the metabolism of carbohydrates (including glycolysis of polysaccharides). Oxygen consumption and carbon dioxide production are also increased; oxygen consumption exceeds the production of carbon dioxide, since oxidation does not always take place until the final formation of carbon dioxide (violation of the Krebs cycle). This leads to accumulation of under-oxidized metabolic products in the inflammation zone, which can be inactivated by C_{60}, optimizing the muscle recovery processes at this stage of the inflammatory process.

During intense physical activities, the flow of oxygen through muscle cells is significantly increased. A high level of oxygen uptake (up to 100-fold) can lead to excessive formation of reactive oxygen species (ROS) and initiate the destruction of functional myofibrils remaining after injury (Ji 1995). In our study, the increased amount of GSH in muscle (without and after therapeutic drugs administration) indicates a compensatory activation of the endogenous antioxidant system to the rapid inflammatory process initiated by muscle injury. Under our experimental conditions, this process is complicated by prolonged non-relaxation contractions of the injured muscle. Many studies have shown that under intense loads there is a significant decrease in the amount of reduced GSH paralleled by an increase in the concentration of its oxidative form (Leeuwenburgh et al. 1997). The described processes occur in the injured muscle with progressive dynamics for at least three days, after which time a recovery period begins.

An increase in H_2O_2 level in muscle injuries leads to increase of CAT activity, which performs a protective antioxidant role, catalyzing the decomposition of hydrogen peroxide into water and oxygen. These results are confirmed both by our studies and by studies carried out earlier in acute experiments on rats (Ji et al. 1992; Leeuwenburgh et al. 1995). At the same time, some studies indicated the absence of any changes in the concentration of CAT in muscles during their motor activity, which can be explained by the absence of decrease in this indicator with the applied therapeutic injections in comparison with other markers (Meydani et al. 1993).

After administration of C_{60} into the injured muscle during fatigue development, the CAT activity significantly decreased compared to fatigue alone. It can be assumed that C_{60}, by affecting the content and activity of endogenous antioxidants, prevent fatigue in actively contracting muscle and, thus, contribute to the maintenance of its normal physiological state (Prylutskyy et al. 2017). However, it should be noted that the level of pathological processes that arose at the first stages of the development of muscle injury exceeded the antioxidant capabilities of C_{60}.

The enhancement of the therapeutic effect of C_{60} in the presence of menthol can be explained by several mechanisms. Thus, studies have shown that menthol has a fast-acting, short-term effect of reducing blood flow, which reduces the level of inflammatory processes. In addition, a single 8 h application of an occlusive patch that contains 3% menthol to treat mild and moderate pain associated with mild and moderate muscle deformity in adult patients significantly alleviates it compared to patients who received placebo, which also affects the level of development of the subsequent inflammatory process (Higashi et al. 2010; Topp et al. 2011). It has been shown that with the internal use of menthol, there is an improvement in muscular performance, mediated by mechanisms associated with its thermal, ventilation, analgesic and stimulating properties (Stevens et al. 2017). Studying the local anaesthetic activity of menthol, the authors have documented its anaesthetic activity in in vivo and in vitro systems (Galeotti et al. 2001). It was also found that the local anaesthetic effect of menthol can be mediated by blockade of sodium channels, which is as effective as the local anaesthetic lidocaine (Haeseler et al. 2002). When an anaesthetic menthol balm is applied to the skin over contracting muscles, the pressor response to static muscle contractions is significantly reduced. This suggests that topical application of menthol has an effect on the responses caused by receptors located in the muscles (Ragan et al. 2004). These facts indicate the ability of menthol to promote the therapeutic properties of C_{60} as an antioxidant in severe pathological muscle injuries and, thus, to alleviate the inflammatory process. In our opinion, C_{60} can affect the activity of endogenous antioxidants, preventing the onset of dysfunction in the active muscle and, thus, maintaining it within the physiological norm during the entire process of muscle activation.

Moreover, we found that menthol can normalize vascular tone (Johnson et al. 2009), which explains, at least in part, the effectiveness of CryoDerm by its effect of TRPM8 cold receptors (https://www.cryoderm.com/index.php?p=392133). There is also accumulating evidence showing the important roles of several TRP subtypes, and notably TRPM8 receptor, in inflammatory and immune cells (Parenti et al. 2016). For example, it was shown that icilin, another selective TRPM8 agonist, reduced inflammation in mice with DSS-induced colitis, likely by inhibiting neuropeptide release (Ramachandran et al. 2013). Thus, TRPM8 agonists are currently considered as novel therapeutic strategies for alleviating intestinal inflammation (Chen et al. 2020). In addition, TRPM8 agonists could inhibit the synthesis of pro-inflammatory cytokines in both human lymphocytes and monocytes (Juergens et al. 2004).

Conclusion

Thus, it was found that injection of C_{60} at a concentration of 1 mg/kg into the damaged muscle of rat improved its contractile function by 25–28%. At the same time, the use of combined injections of C_{60} and menthol (at the concentration 1 mg/kg) improved this index by additional 27–39% and simultaneously stabilized the decrease in muscle strength observed throughout the experiment. A tendency towards a decrease in the indexes of the used biochemical parameters (creatinine, CPK, LA, LDH, TBARS, H_2O_2, GSH and CAT) by 10–15% were found with the therapeutic administration of C_{60}. With combined injections of C_{60} and menthol, these biochemical parameters decreased by an additional 17–24%.

The discovered positive changes in the biomechanical and biochemical parameters of the functioning of the injured skeletal muscle open up the prospect of using an aqueous solution of C_{60} in combination with menthol in low doses (1 mg/kg) as effective combinational therapy capable of correcting the pathological state of skeletal muscle following its mechanical injury.

Author contributions DN, TM and VS: biomechanical analysis. OV and KB: biochemical analysis. PS and UR: preparing of the samples. AZ and YP: coordination the research work, analysis of the data and preparing of the manuscript.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

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Analysis of Biomechanical and Biochemical Markers of Rat *Muscle Soleus* Fatigue Processes Development during Long-Term Use of C_{60} Fullerene and N-Acetylcysteine

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Abstract: The development of an effective therapy aimed at restoring muscle dysfunctions in clinical and sports medicine, as well as optimizing working activity in general remains an urgent task today. Modern nanobiotechnologies are able to solve many clinical and social health problems, in particular, they offer new therapeutic approaches using biocompatible and bioavailable nanostructures with specific bioactivity. Therefore, the nanosized carbon molecule, C_{60} fullerene, as a powerful antioxidant, is very attractive. In this study, a comparative analysis of the dynamic of *muscle soleus* fatigue processes in rats was conducted using 50 Hz stimulation for 5 s with three consistent pools after intraperitoneal administration of the following antioxidants: C_{60} fullerene (a daily dose of 1 mg/kg one hour prior to the start of the experiment) and N-acetylcysteine (NAC; a daily dose of 150 mg/kg one hour prior to the start of the experiment) during five days. Changes in the integrated power of muscle contraction, levels of the maximum and minimum contraction force generation, time of reduction of the contraction force by 50% of its maximum value, achievement of the maximum force response, and delay of the beginning of a single contraction force response were analyzed as biomechanical markers of fatigue processes. Levels of creatinine, creatine phosphokinase, lactate, and lactate dehydrogenase, as well as pro- and antioxidant balance (thiobarbituric acid reactive substances, hydrogen peroxide, reduced glutathione, and catalase activity) in the blood of rats were analyzed as biochemical markers of fatigue processes. The obtained data indicate that applied therapeutic drugs have the most significant effects on the 2nd and especially the 3rd stimulation pools. Thus, the application of C_{60} fullerene has a (50–80)% stronger effect on the resumption of muscle biomechanics after the beginning of fatigue than NAC on the first day of the experiment. There is a clear trend toward a positive change in all studied biochemical parameters by about (12–15)% after therapeutic administration of NAC and by (20–25)% after using C_{60} fullerene throughout the experiment. These findings demonstrate the promise of using C_{60} fullerenes as potential therapeutic nanoagents that can reduce or adjust the pathological conditions of the muscular system that occur during fatigue processes in skeletal muscles.

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1. Introduction

One of the most important characteristics of the human muscular system, along with the magnitude of force generated by skeletal muscles, is their ability to maintain the level of effort generation that was given by the central nervous system (CNS) over a period of time. Muscle fatigue occurs when contraction-inducing factors continue to flow to the muscle with constant intensity, and the level of strength generated by the muscle itself gradually decreases. Currently, there is no single separate mechanism of muscle fatigue development, and there is a set of mechanisms at different system levels [1]: in particular, disorders of the CNS [2], dysfunction of peripheral nerves and neuromuscular junctions [3], physiological reversible changes directly in the skeletal muscles [4,5] that perform the work.

Accumulated data evidence that disruption of the "excitation–contraction" coupling is most likely localized in muscle fibers; this explains the fatigue-induced decrease in maximum strength in humans, while central (neural) fatigue plays a greater role in the event of an inability to continue sustained low-intensity contraction [6]. Based on data from intact single muscle fibers, fatigue-induced impairment of "excitation–contraction" includes: a decrease in the number of active cross-bridges due to the reduction of Ca^{2+} ions release; a decrease of myofilaments sensitivity to Ca^{2+}; reduction of the force generated by each active cross-bridge.

Calcium ions play a critical role as initiators and preservatives of the cross-bridging cycle in the formation of skeletal muscle strength. The authors of [7] presented a new chemo-mechanical model for analyzing the role of Ca^{2+} in muscle fatigue, as well as predicting muscle fatigue. It is assumed that even minor disturbance of myocytes integrity, imbalance of "Ca^{2+}—muscle pathology" can play an important role in the rate and degree of development of fatigue processes [7,8].

Deceleration of the contractile properties of skeletal muscles is one of the characteristic signs of fatigue [9]. There are three factors that contribute to the loss of power by mammalian muscles at physiological temperatures: a decrease in isometric strength, which mainly indicates a decrease in the number of active cross-bridges, a slowdown in the maximum speed of unloaded shortening, and an increase in the curvature of the force-velocity ratio [9]. This last change is the main reason for the loss of muscle power during the development of pathologies associated with changes in the integrity of the myocyte membrane complex [10].

The dynamic of the contractile component is determined by subtle mechanisms of interaction between motor neuron pools that occur in muscle via activated motor neuron and activation of the interaction between actin and myosin filaments. Under isometric conditions, the analysis of the recorded force developed by the muscle during frequency-modulated stimulation of its nerve is a qualitative indicator of the level of myopathic pathological processes [11]. Rapid processes of excitation of the contractile apparatus in the process of long-term activation of the muscle fiber usually undergo a slow and steady modification, which may partly be associated with phosphorylation of myosin light chains located in the neck of the bridge. A slower process of dephosphorylation under conditions of prolonged continuous activation of the muscle fiber causes stable phosphorylation of myosin, which, apparently, increases the mobility of the bridges or changes their orientation [12]. Analysis of the amplitude-velocity changes in the force response (biomechanical markers of the contractile process) of the activated muscle gives the possibility to assess the level of influence of the developing pathology on these processes [13]. These processes play an important role in precise positioning movements of the hand and fingers: even minor disturbances in the control system of these movements lead to very serious physiological problems [14].

Literature data indicate that free radicals are an important pathogenic factor in the process of muscle fatigue [15,16]. They include initiation of lipid peroxidation (LPO), direct inhibition of mitochondrial respiratory chain enzymes and ATPase activity, inactivation of glyceraldehyde-3-phosphate dehydrogenase and membrane sodium channels, etc. [17,18]. One of the mechanisms by which free radicals cause tissue damage is the interaction of the hydroxyl radical with the hydrogen atoms of the methyl groups of polyunsaturated fatty acids. This process initiates POL, which, in turn, leads to the increase in permeability of cell membranes [19].

The ability of the biocompatible C_{60} fullerenes and their derivatives to inactivate the reactive oxygen species (ROS) was first demonstrated by Krustic et al. [20]. It has been established that pristine C_{60} fullerenes have a dose-dependent protective effect against oxidative-mediated muscle trauma [21,22]. Moreover, C_{60} fullerene protects the rat's liver from ROS [23,24]. Given the accumulated data about the powerful antioxidant properties of C_{60} fullerenes [25], the purpose of this work was to conduct a comparative analysis of changes in biomechanical and biochemical markers of *muscle soleus* fatigue processes development in rats during long-term therapeutic use of C_{60} fullerene and the well-known antioxidant N-acetylcysteine (NAC) [26].

2. Materials and Methods

2.1. Preparation of C_{60}FAS

For the preparation of C_{60} fullerene aqueous colloid solution (C_{60}FAS) at a maximum concentration of 0.15 mg/mL, we used a saturated solution of pristine C_{60} fullerene (purity > 99.96%) in toluene with a C_{60} 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. The procedure was continued until the toluene had completely evaporated and the water phase became yellow colored. Filtration of the aqueous solution allowed to separate the product from undissolved C_{60} fullerenes [27,28]. The prepared C_{60}FAS is stable within 12–18 months at temperature +4 °C.

2.2. AFM and STM Analysis

The atomic force microscopy (AFM) and scanning tunneling microscopy (STM) were performed to determine the size of C_{60} fullerene particles (their aggregates) in an aqueous solution. Measurements were done with the "Solver Pro M" system (NT-MDT, Moscow, Russia). A drop of investigated solution was transferred to the atomic-smooth substrate to deposit layers. Measurements were carried out after complete evaporation of the solvent. For AFM studies, a freshly broken surface of mica (SPI supplies, V-1 grade) was used as a substrate. Measurements were carried out in a semicontact (tapping) mode with AFM probes of the RTPESPA150 (Bruker, 6 N/m, 150 kHz) type. STM studies were performed with the Au (111) surface obtained after annealing substrates of Au/mica (Phasis, Geneva, Switzerland) in a gas burner flame (propane-butane). The typical tunneling current and voltage values were 0.027–0.1 nA and 0.1–1 V, respectively.

2.3. Animals

Male Wistar rats (170 ± 12 g, 2-month-old) were bred and housed in standard temperature conditions (21–23 °C), a lighting (12/12 h light-dark cycle), at humidity (30–35)%. All animals had unlimited access to chow and tap water. The study was carried out in strict accordance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986) and was approved by the Bioethical Committee of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv, Ukraine.

Four experimental groups of animals (n = 7 in each group) were studied: after C_{60}FAS and NAC administration, which were compared with the control group ("fatigue", no drug administration) and the intact group.

Based on our previously obtained data [11,29], the research protocol involved intraperitoneal injection of C_{60}FAS and NAC at a daily dose of 1 and 150 mg/kg, respectively, one hour before the experiment for 5 days.

It is important to note that water-soluble C_{60} fullerenes at low concentrations did not manifest any toxic effects as to normal cells [30,31]. Moreover, the selected dose of C_{60}FAS in our experiments is significantly lower than the LD_{50} value, which was 600 mg/kg body weight when administered orally to rats [23] and 721 mg/kg when administered intraperitoneally to mice [31].

2.4. Biomechanical and Biochemical Analysis

The object of the study was the rat *muscle soleus*. In preliminary preparation for the experiment, anesthesia was performed by intraabdominal injection of nembutal (40 mg/kg). Standard preparation included cannulation (*a. carotis communis sinistra*) for pressure measurement and laminectomy at the lumbar spinal cord level. *Muscle soleus* was released from surrounding tissues, and their tendon parts were connected to force measurement sensors in the distal part. To prepare for modulated efferent stimulation, the ventral roots in the respective segments were transected directly at their exit points from the spinal cord.

The dynamic properties of muscle contraction were studied under conditions of muscle activation using the method of modulated efferent stimulation [32]. Fatigue was induced by successive stimulation impulses with a frequency of 50 Hz and a duration of 5 s each, without a relaxation period between them. The sum of such stimulation signals was 500 s, followed by 5 min of relaxation. The number of stimulation pools was three. The external load on the muscle was controlled using a system of mechanostimulators. Changes in contraction force were measured by strain gauges. During the analysis of the results, we used the following quantitative parameters: integrated muscle power, levels of maximum and minimum strength generation of contraction, time of reduction of the contraction force by 50% of its maximum value, achievement of the maximum force response, and delay of the beginning of single contraction force response.

The levels of creatinine, creatine phosphokinase (CPK), lactate (LA), lactate dehydrogenase (LDH), thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H_2O_2), reduced glutathione (GSH), and catalase (CAT) activity as markers of muscle injury [33] were determined in the blood plasma of experimental animals using clinical diagnostic equipment—a haemoanalyzer [21].

2.5. Statistical Analysis

Statistical processing of the measurement results was performed by methods of variational statistics using the software Origin 9.4. Each of the experimental force curves obtained in the work is the result of averaging 10 similar experiments. At least three repetitions were performed for each biochemical measurement. Data are expressed as means ±SEM for each group. Differences from experimental groups were indicated by one-way ANOVA described by Bonferroni's multiple comparison test. Values of $p < 0.05$ were considered significant.

3. Results and Discussion

3.1. Characterization of C_{60}FAS

The AFM images of the C_{60} fullerene layers show casually placed point-shaped objects up to 10 nm high, mostly 0.7–3.5 nm (Figure 1a). The height of the smallest objects 0.7 ± 0.2 nm) agrees well with the molecular diameter of C_{60} fullerene, which allowed them to be identified as individual molecules. Larger objects correspond to C_{60} fullerene bulk clusters. We analyzed the statistics of the distribution of objects by height according to data on 100 randomly selected molecules or clusters within a certain area of the surface. Depending on the height, they were included in one of the size groups from 0.7 ± 0.2 nm to 3.5 ± 0.2 nm, which corresponds to one to five diameters of the C_{60} molecule. According to statistics, the relative number of single C_{60} molecules was 60%, clusters with a height of

~1.3 nm—28%, and clusters of other size groups (~2.0 nm, ~2.8 nm, and ~3.5 nm)—4% each. The clusters had symmetrical bell-shaped Z-profiles with sharp maxima in the middle. This indicates that they were formed in solution long before application to the substrate.

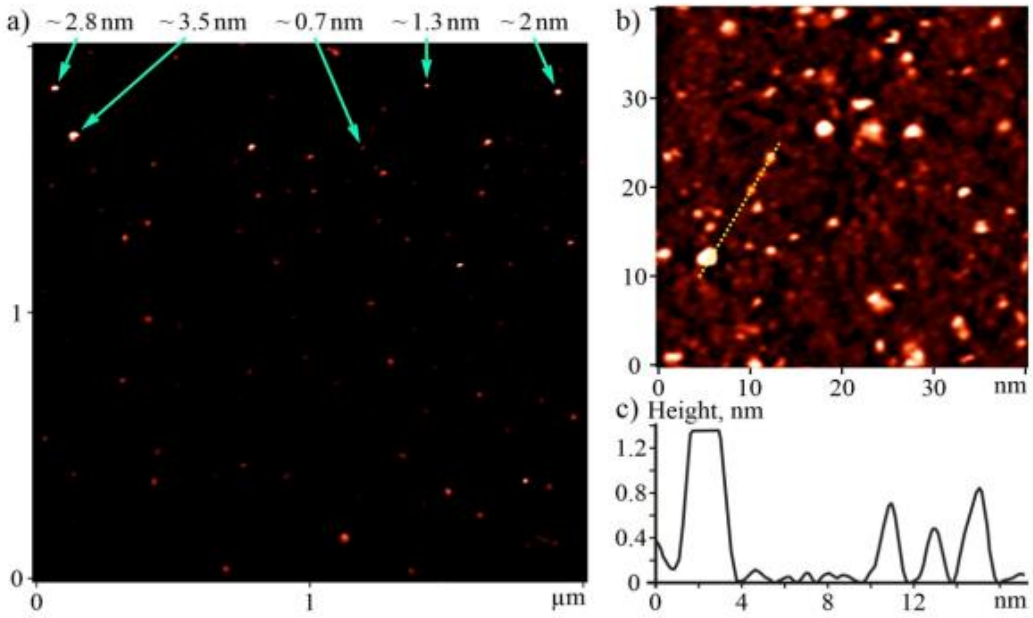

Figure 1. AFM image of C_{60} fullerene layer deposited from C_{60}FAS on a mica substrate (**a**). The values near the arrows indicate the height of the nanoobjects including single C_{60} fullerene (~0.7 nm); STM image of C_{60} fullerene layer deposited from C_{60}FAS on an Au(111) substrate (**b**). Z-profile along the dashed line marked on the STM image (**c**).

The STM method allowed us to more accurately determine the lateral sizes of objects. On STM images, the majority of objects also had a point-like shape and a height of 0.75 ± 0.15 nm or 1.3 ± 0.2 nm (Figure 1b,c), which is consistent with the value of one or two molecular diameters of C_{60} fullerene. Among them, the relative part of the former was ~80%, and the width of their profiles at half height was 1.5–1.8 nm (Figure 1c). For STM, the minimum possible tip radius is equal to the radius of its closest atom to the surface, to which the width of the tunnel gap (~0.2 nm) should be added. Our STM probe was made of an alloy of platinum and iridium, whose atomic radii are ~0.18 nm. Thus, C_{60} molecules, which can be modeled as spheres with a diameter of ~0.7 nm, will have a lateral size of at least $0.7 + 2 \times (0.2 + 0.18) =$ ~1.5 nm on STM images. This proves that the objects visible in the images with a height of ~0.75 nm and a diameter of 1.5–1.8 nm are individual molecules. Objects of greater height correspond to clusters of C_{60} fullerene. Analysis of the profiles showed that their shape is close to spherical. The existence of such aggregates in aqueous solutions was predicted earlier theoretically [34].

Thus, the data of both methods (AFM and STM) indicate that C_{60} fullerene was in the non-aggregated or low-aggregated state in the studied solutions. The isolated arrangement of C_{60} molecules (clusters) is explained by the existence of electrostatic repulsion forces between them, namely they demonstrated a high negative surface charge (zeta potential value was −25.3 mV at room temperature [31]), indicating a very low tendency for aggregation in aqueous solution (i.e., a high solute stabilization).

3.2. Biomechanical Analysis

Despite the emergence of new experimental approaches to the analysis of neuromuscular regulation processes at the microlevel, traditional electrophysiological models using neuromuscular preparation in vivo are of paramount importance [4]. These studies should be carried out not only for the purpose of a more accurate quantitative analysis of the pathologies of muscle dynamics but also for a detailed study of the aggregation of the central processes involved in the regulation of muscle contraction. Figure 2 shows the change in contractile strength of rat *muscle soleus* after application of 50 Hz stimulation for 5 s in three successive pools for 500 s each with 5 min of relaxation between them after C_{60}FAS and NAC administration during 5 days.

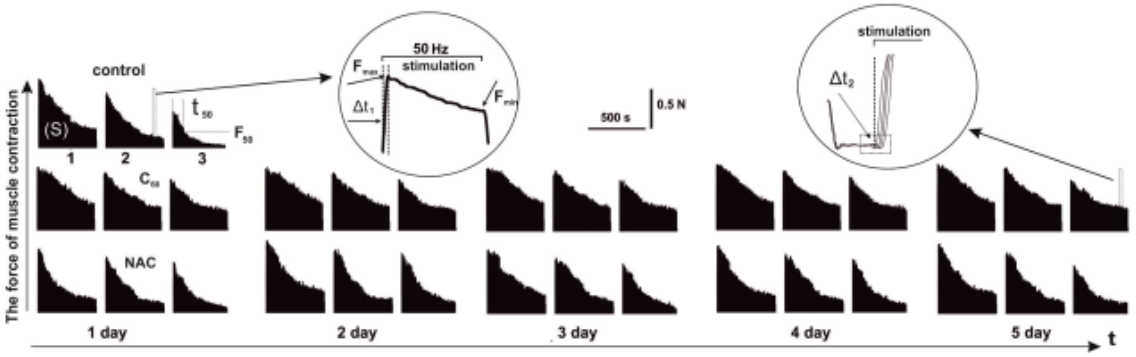

Figure 2. Recording the contractile force of rat *muscle soleus* after application of 50 Hz stimulation for 5 s in three consecutive pools (1,2,3) for 500 s each with 5 min relaxation between them: native muscle (control) and muscle after C_{60}FAS (C_{60}) and NAC administration during 1, 2, 3, 4, and 5 days. S—integrated muscle power (calculated area under the power curve); F_{max} and F_{min}—maximum and minimum strength of a single contraction; Δt_1—the time to reach the maximum force of a single muscle contraction; Δt_2—time of muscle force response beginning; t_{50}—time of decreasing the muscle contraction force by 50% (F_{50}) of the maximum value in the contraction pool.

Several basic biomechanical parameters were taken into consideration for analyzing the miotic response of the studied muscle [35]. The presence of changes in each of them indicates dysfunction of a certain link in the "excitation-response of the muscle preparation" chain.

Change in the integrated power of muscle contraction. Integrated power, as the calculated area under the power curve (Figures 2 and 3), is an indicator of the overall performance of the muscle with the applied stimulation pools [22]. The analysis of this parameter makes it possible to evaluate the kinetics of the formation of muscle fatigue in the "force–external load" equilibrium system.

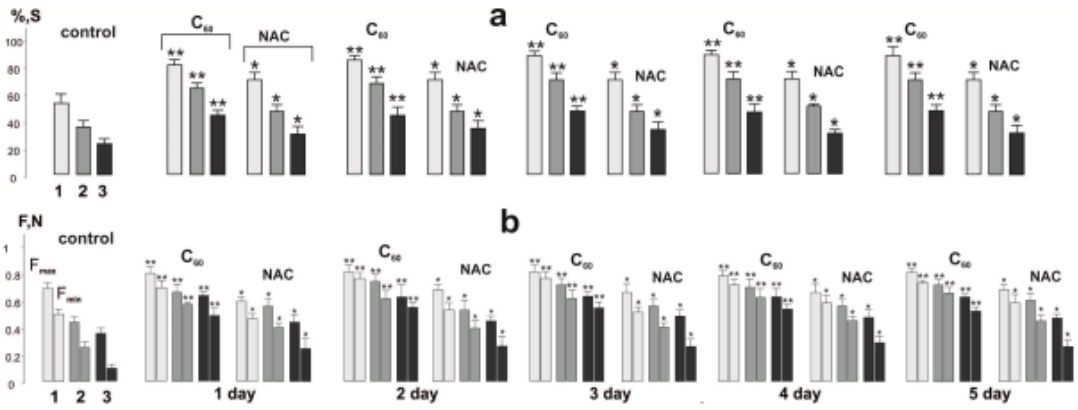

Figure 3. The integrated power of the rat *muscle soleus* (S, presented as a percentage of the maximum values) (**a**) and the peak values of its contraction force (F, N) (**b**) after applying 50 Hz stimulation for 5 s in three consecutive pools (1,2, 3) for 500 s each with 5 min relaxation between them: native muscle (control); muscle after administration of C_{60}FAS (C_{60}) and NAC for 1, 2, 3, 4, and 5 days. F_{max} and F_{min} are the maximum and minimum forces of a single muscle contraction. * $p < 0.05$ compared to control; ** $p < 0.05$ compared to values in the NAC group.

The change in the integrated power of rat *muscle soleus* (Figures 2 and 3) showed its significant decrease already after 1st stimulation pool, which amounted to 58 ± 4%. After the relaxation period, the integrated power progressively decreased at the 2nd and 3rd stimulation pool, which was 39 ± 2% and 24 ± 3%, respectively.

The use of NAC increased the value of this indicator to 77 ± 5%, 50 ± 5%, and 31 ± 3% at 1, 2, and 3 stimulation pools, respectively. Thus, the therapeutic effect of NAC was 32%, 28%, and 25% at 1st, 2nd, and 3rd stimulation pools, respectively (compared to control). Using of NAC injections during the next 4 days did not show significant changes in integrated power.

Injection of C_{60}FAS led to an increase in the level of this parameter to 82 ± 4%, 66 ± 5%, and 42 ± 3% at 1, 2, and 3 stimulation pools, respectively. Thus, the protective effect after the first injection of C_{60}FAS was 41 ± 3%, 69 ± 6%, and 75 ± 3% at 1st, 2nd, and 3rd stimulation pools, respectively (compared to control). There is a progressive increase in the therapeutic effect of C_{60}FAS throughout the experiment. So, on the 5th day of C_{60}FAS therapy, the indicators of integrated power were 89 ± 3%, 77 ± 3%, and 50 ± 3% at the 1st, 2nd, and 3rd stimulation pool, respectively, which corresponds to an increase in its effect by 8%, 16%, and 19% compared to a single injection. It should be noted that a further increase in the duration of C_{60}FAS application did not lead to statistically significant changes in muscle biomechanics and, therefore, is not presented in this work. In our opinion, this may be due to the establishment of the maximum possible equilibrium concentration of C_{60} fullerene in the active muscle on the 5th day of its use.

Changing the generation levels of maximum (F_{max}) and minimum (F_{min}) contraction force. The F_{max} marker is an indicator of the general dysfunction of the muscular system, namely, an indicator of a decrease in the maximum possible force response during the development of fatigue (Figures 2 and 3). A change in this parameter can be associated with both the development of fatigue processes in the neural component and the miotic components of the studied pathology [36]. Its dysfunction, in our opinion, can also be due to a violation of the integrity of the signals that generate motor neurons into the synaptic current, which leads to a violation of the summation of transmembrane currents in accordance with the internal membrane properties. All this affects the pathological transformation of the sequence of action potentials that trigger muscle contraction, causing a maximum force response.

The F_{min} marker is an indicator of the maximum pathological changes during the development of fatigue processes in each successive contractile act (Figures 2 and 3). While

performing simple single-joint movements, this marker is the main indicator of muscle dysfunction, the phenomenological analysis of which makes it possible to establish the presence of causal relationships between the level of decrease in muscle biomechanical activity, the main mechanical parameters of movements, and the level of development of the pathological process. If there is a difference between the maximum and minimum force response of an active muscle with a constant frequency stimulation signal, there are serious difficulties in correcting the control of muscle strength by the CNS, which, in turn, makes it difficult to correct the precise positioning of the joints, which is observed during the development of muscle fatigue.

The analysis of the obtained mechanograms showed that the maximum force indicators of contraction were 0.70 ± 0.08 N, 0.42 ± 0.05 N, and 0.39 ± 0.05 N at 1st, 2nd, and 3rd stimulation pools, respectively, in the control measurements (Figure 3). Thus, the decrease in maximum strength was 40% and 55% of the initial values at the 2nd and 3rd contraction pools, respectively.

The use of NAC did not significantly change the maximum contraction force of any of the three studied stimulation pools during a five-day application.

Injections of C_{60}FAS resulted in a change in maximum contraction force to 0.81 ± 0.10 N, 0.65 ± 0.05 N, and 0.63 ± 0.05 N at 1st, 2nd, and 3rd stimulation pools, respectively, after the first injection. Thus, the therapeutic effect of C_{60}FAS was 15%, 54%, and 63% at 1st, 2nd, and 3rd contraction pools, respectively. The therapeutic effect of C_{60}FAS for five days led to the following change in the above indicator: 0.82 ± 0.10 N, 0.75 ± 0.05 N, and 0.64 ± 0.05 N at 1st, 2nd, and 3rd pools stimulation, respectively. As noted, there is practically no difference in the maximum contraction force after using C_{60}FAS at all stimulation pools, in contrast to an almost 50% decrease in this indicator in the control values. Thus, this therapy supports the stabilization of the mechanokinetics of the contractile process during prolonged activations of the studied muscle.

The minimum indicators of the force response of the studied muscle showed its decrease to 0.50 ± 0.04 N, 0.21 ± 0.03 N, and 0.14 ± 0.01 N at 1st, 2nd, and 3rd stimulation pools, respectively, in control measurements (Figure 3). It should be noted that at the 3rd contraction pool, the decrease in the minimum force was five times less than the initial value, which indicates the presence of very significant fatigue processes in the muscle due to an insufficient relaxation period.

Injections of NAC increased the minimum strength values, which were 0.57 ± 0.05 N, 0.41 ± 0.05 N, and 0.27 ± 0.02 N at 1st, 2nd, and 3rd stimulation pools, respectively. Thus, the application of NAC significantly corrected the minimum force of contraction only at the 2nd and 3rd pool of contractions. At the same time, the five-day use of this drug did not significantly increase the indicators of the minimum contraction force.

C_{60}FAS injections significantly changed the mechanokinetics of the contractile process: the minimum force was 0.72 ± 0.05 N, 0.59 ± 0.06 N, and 0.51 ± 0.05 N at 1st, 2nd, and 3rd stimulation pools, respectively, after the first injection. On the 5th day of C_{60}FAS administration, these indicators were 0.79 ± 0.06 N, 0.65 ± 0.05 N, and 0.59 ± 0.05 N at 1st, 2nd, and 3rd pools, respectively. Thus, the therapeutic effect of C_{60}FAS was 58%, 209%, and 421% after a single application and increased by (13–16)% after a five-day administration. Thus, the maximum effects of C_{60}FAS application are observed at the 3rd contraction pool, which, in turn, shows the strongest violations of contraction biomechanics during the development of skeletal muscle fatigue.

Change in the time of contraction force decrease in muscle soleus by 50% of its maximum value (t_{50}). We analyzed the time of decrease in rat *muscle soleus* contraction force by 50% of its maximum value in each of the three stimulation pools, which made it possible to assess the development of fatigue in different time ranges (Figure 4). In fact, this parameter is a marker of the "activation" of adaptive mechanisms that prevent the onset of progressive fatigue.

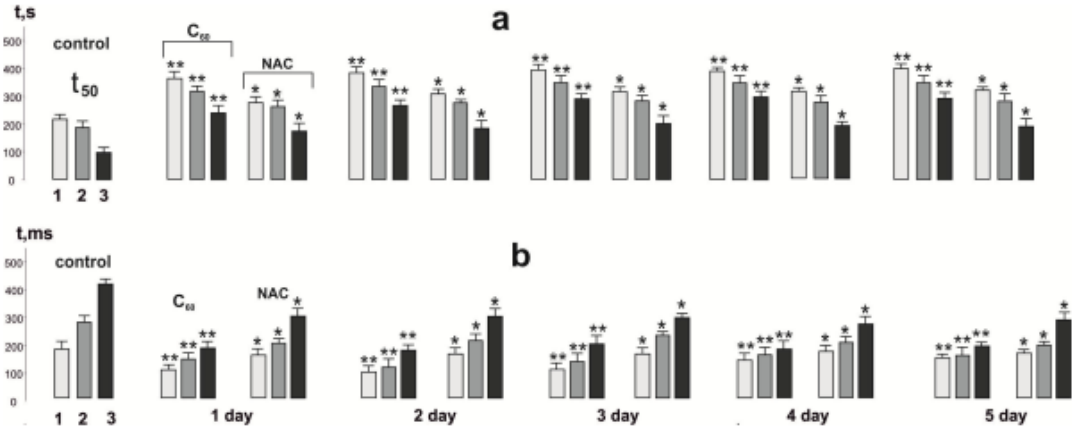

Figure 4. Time of contraction force decreasing in *muscle soleus* by 50% of its maximum value (t_{50}) in each of the three pools of contractions (1,2,3) (**a**) and the time of reaching the maximum force of a single contraction of the muscle (**b**) after using 50 Hz stimulation for 5 s in three consecutive pools lasting 500 s each with 5 min relaxation between them: native muscle (control); muscle after injection of C_{60}FAS (C_{60}) and NAC for 1, 2, 3, 4 and 5 days. * $p < 0.05$ compared to control; ** $p < 0.05$ compared to NAC group.

In the control, this indicator was 210 ± 12 s, 190 ± 8 s, and 104 ± 6 s at 1st, 2nd, and 3rd pools, respectively. According to the presented data, the most significant violations (more than 200%) occur in the 3rd pool of the contractile process.

After NAC application, these values were 282 ± 10 s, 275 ± 7 s, and 180 ± 7 s, which is a significant indicator of the therapeutic effect of this drug on the studied marker (33%, 44%, and 81%, respectively). It should be noted that NAC produces the maximum effect at the 3rd stimulation pool and, as in previous studies, does not change its effect during five days of use.

The use of C_{60}FAS changed the time of decrease in the contraction force of rat *muscle soleus* by 50% of its maximum value, which was 380 ± 14 s, 310 ± 11 s, and 260 ± 16 s at 1st, 2nd, and 3rd pools, respectively, after the first injection. In percentage terms, these indexes were 180%, 163%, and 260%, respectively. Use of C_{60}FAS during five days increased these indicators by (14–18)%.

Change in the time of reaching the maximum force response. High-frequency stimulation of peripheral afferents that form monosynaptic contacts with a motor neuron causes an effective summation of successive action potentials and a stable depolarization of the cell membrane [37]. In this case, the impulse frequency is determined by the average level of membrane depolarization and increases with a rise in stimulation frequency. With the development of fatigue processes in the muscle, a characteristic adaptive decrease in the time of stimulus conduction through the nervous tissue and an increase in the latent period preceding the onset of muscle force generation become noticeable. A change in this indicator is a characteristic marker of the presence of pathological processes in the neuromuscular preparation associated with the triggering of the beginning of the interaction of the myocyte actin-myosin complex [37]. Under the conditions of this experiment, the time to achieve the maximum force response during the development of fatigue processes in the control was 190 ± 12 ms, 293 ± 17 ms, and 419 ± 27 ms at 1st, 2nd, and 3rd stimulation pools, respectively (Figure 4).

After NAC injection, these values were 181 ± 15 ms, 208 ± 12 ms, and 310 ± 11 ms, respectively. Thus, the therapeutic effect was 5%, 30%, and 29%, respectively. As well as the analogous markers, this indicator showed significant differences only at the 2nd and 3rd stimulation pool and did not change after five days of NAC use.

The use of C_{60}FAS changed this indicator by 108 ± 12 s, 152 ± 17 s, and 191 ± 12 s at 1st, 2nd, and 3rd pools, respectively, after the first injection. In percentage terms, these values were 75%, 92%, and 119%, respectively. It is important to note that the five-day use of C_{60}FAS did not significantly change this indicator, which may be due to the maximum saturation of the active muscle with fullerene C_{60} already on the first day of its use to achieve the maximum strength response and establish the optimal concentration range.

Change in time delays the force response beginning of a single contraction. The time parameters of the conduction of stimulation pools along the axon do not remain constant either with a change in the intensity of stimulation or in the relaxation time. The study of changes in time delays in the conduction of impulses with an increase in the number of stimuli allowed us to assess the level of pathological processes in the neuromuscular preparation during long-term reactions of the muscular system. In a detailed analysis of the development of pathological processes associated with the development of skeletal muscle fatigue, it is necessary to use long-term stimulation pools that cause long-term transsynaptic activation of motor neurons [38]. At the same time, a characteristic adaptive decrease in the time of stimulus conduction through the nervous tissue becomes noticeable, induced by the development of fatigue processes. The change in this indicator is a characteristic marker of the presence of pathological processes in the neuromuscular preparation after using stimulation signals close to physiological parameters.

Figure 5 shows mechanograms of 10 contractions that were taken sequentially at equal time intervals from each stimulation pool using 50 Hz stimulation for 5 s in three consecutive pools, showing changes in the time of the beginning of rat *muscle soleus* force response. In the control, the value of the time delay for the beginning of the force response was (31–38) ± 3 ms for 1–10 single contractions at the 1st stimulation pool and progressively increased to (36–45) ± 2 ms, (42–74) ± 4 ms at the 2nd and 3rd pools, respectively. Thus, the increase in the time delay of force response beginning at the last contraction of the 3rd stimulation pool was about 100%.

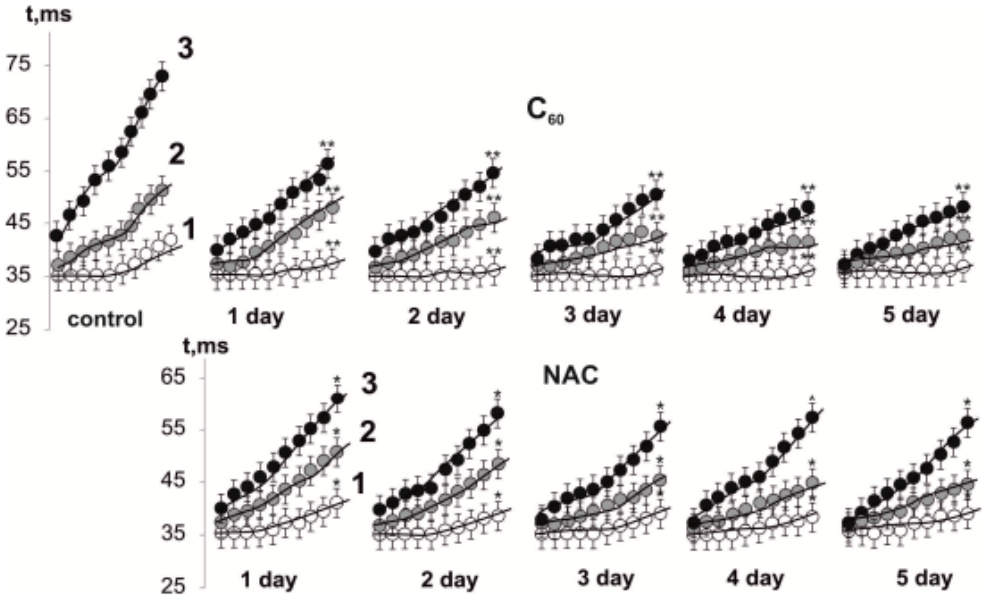

Figure 5. Time delay of force response beginning of 10 single contractions in rat *muscle soleus* after applying 50 Hz stimulation for 5 s in three consecutive pools (1, 2, 3) of 500 s each with 5 min of relaxation between them: native muscle (control); muscle after injection of C_{60}FAS (C_{60}) and NAC for 1, 2, 3, 4, and 5 days. * $p < 0.05$ compared to control; ** $p < 0.05$ compared to NAC group.

These values were (35–34) ± 1 ms, (36–43) ± 2 ms, and (39–59) ± 4 ms, respectively, at 1st, 2nd, and 3rd stimulation pools after NAC injection. Thus, the therapeutic effect was about 25% only in the 3rd stimulation pool. Five-day therapy with this drug did not show significant differences.

The use of C_{60}FAS also changed this indicator: (35–36) ± 2 ms, (37–42) ± 5 ms, and (39–48) ± 5 ms, respectively, which amounted to about 54% of the therapeutic effect at the 3rd stimulation pool. Five-day use of C_{60}FAS increased this indicator by another (8–12)%.

3.3. Biochemical Analysis

The change in the chemical composition of the blood during the development of fatigue processes is a reflection of the biochemical shifts that occur in the skeletal muscle during active work [39]. Therefore, the analysis of the biochemical composition of the blood provides both a direct assessment of the biochemical changes that occur in the muscle during prolonged contraction and the ability to evaluate the therapeutic effect of the drug used on pathological processes. Selected for the study biochemical indicators such as the levels of creatinine, CPK, LA, and LDH are markers of physiological disorders of muscle tissue due to the development of fatigue and related dysfunctions of the muscular apparatus (Figure 6).

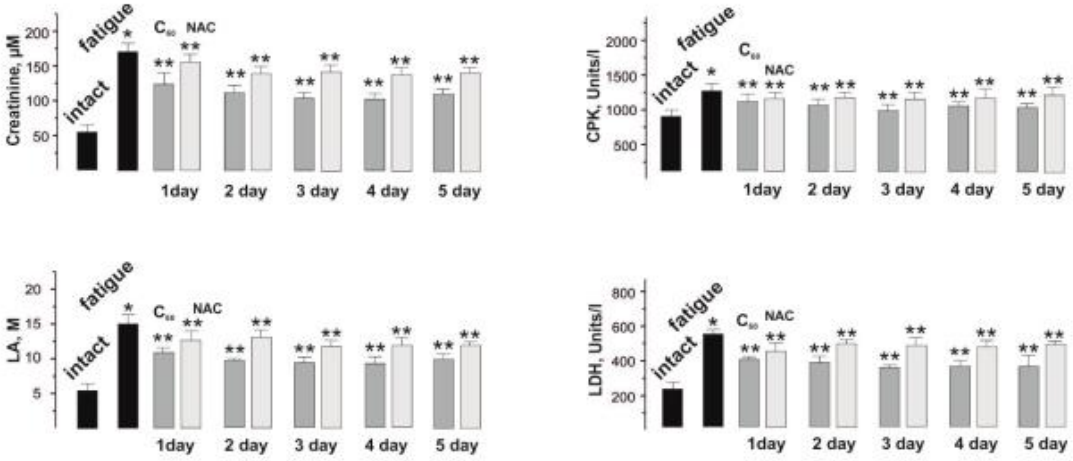

Figure 6. Biochemical indicators of the fatigue processes development in rat *muscle soleus*: the levels of creatinine, CPK, LA, and LDH in the blood after application of three-component stimulation and administration of C_{60}FAS (C_{60}) and NAC for 1, 2, 3, 4, and 5 days. * $p < 0.05$ relative to intact group; ** $p < 0.05$ relative to the fatigue group (without drugs).

A change in the level of creatinine, a product formed in muscles during the destruction of intramuscular structures while prolonged active work, makes it possible to assess the level of damage to myocytes during prolonged contractions. This indicator increased from 50 ± 2 μM in the intact group to 169 ± 5 μM after the application of three-component stimulation.

The use of NAC reduced this indicator to 152 ± 2 μM after a single injection and reduced its value by no more than 3% during five days of therapy with this drug.

The use of C_{60}FAS reduced this indicator to 122 ± 2 μM after a single injection and reduced its value by no more than 7% during a five-day therapy with this drug. A significant decrease in the creatinine fraction (27% of therapeutic effect), in our opinion, is caused by the protective effect of C_{60} fullerenes, its molecules protect the membranes of skeletal muscle cells from nonspecific free radical damage by actively absorbing free radicals.

CPK is an enzyme found in high concentration in skeletal muscle. With mechanical damage that occurs during prolonged muscle activity, this enzyme is released from the cells

with a further increase in its level in the blood. The increase in the CPK fraction in the blood during the experiment from 960 ± 13 Units/L in the intact group to 1280 ± 22 Units/L is the result of a cascade physiological violation of the integrity of the walls of myocytes, which increases with active long-term relaxation-free contraction.

Injections of NAC and C_{60}FAS led to a decrease in this enzyme to 1280 ± 24 Units/L and 1092 ± 27 Units/L, respectively, and a slight increase in the therapeutic effect of C_{60}FAS (8%) was observed only by the 5th day of the experiment.

In an active muscle, most metabolic processes occur under anaerobic conditions, as a result of which the muscle uses a large number of mitochondrial enzymes and, as a result, there is an accumulation of a large amount of LA, which does not have time to be oxidized during prolonged muscle stimulation [39]. An increase in the level of lactic acid in an active muscle indicates that the amount of its entry into the cell exceeds the amount of its oxidation and output. In the intact group, the LA level was 5.0 ± 0.4 M. After fatigue initiation, its value increased to 16 ± 1 M.

Injections of NAC and C_{60}FAS reduced lactate levels to 13 ± 1 M and 11 ± 1 M, respectively. Five-day C_{60}FAS therapy reduced the LA level to 9.0 ± 0.5 M. Thus, C_{60}FAS therapy led to an increase in LA oxidation by almost 40% compared to the control ("fatigue"), which turned out to be more effective than NAC 35%.

The level of change in LDH, an enzyme that catalyzes the oxidation of lactic acid, made it possible to assess the general state of muscle performance after the beginning of fatigue. The change in the level of this enzyme from 210 ± 11 Units/L in the intact group to 540 ± 12 Units/L after induced fatigue is evidence of the development of significant muscle dysfunctions associated with an excess of fatigue pathogens.

Injections of NAC reduced the content of LDH to 490 ± 11 Units/L and its value did not change significantly during the five days of the experiment.

Injection of C_{60}FAS reduced the level of LDH to 400 ± 11 Units/L after the first administration and to 380 ± 11 Units/L on the 5th day of its use.

Inflammatory cascade processes that occur immediately after the initiation of fatigue in the skeletal muscle are a source of ROS and contribute to the intensification of LPO processes [7]. This prevents the muscles from adequately performing work and significantly increases the length of the recovery period. The data obtained clearly demonstrate an increased level of markers of peroxidation and oxidative stress (CAT, H_2O_2, TBARS, and GSH) after the beginning of muscle fatigue and their decrease due to the applied therapy (Figure 7).

Thus, CAT activity increased from 0.9 ± 0.1 mM/min in the intact group to 3.2 ± 0.1 mM/min after the development of muscle fatigue.

Injections of NAC and C_{60}FAS reduced CAT activity to 2.7 ± 0.1 mM/min and 2.5 ± 0.1 mM/min, respectively, during the course of the experiment.

The level of H_2O_2 was 3.1 ± 0.2 mM during the development of fatigue (0.8 ± 0.1 mM in the intact group) and 2.4 ± 0.2 mM and 2.1 ± 0.2 mM after the administration of NAC and C_{60}FAS, respectively, during the experiment.

The change in TBARS level was 5.8 ± 0.2 μM during the development of fatigue (2.5 ± 0.3 μM in the intact group) and 4.3 ± 0.1 μM and 4.1 ± 0.4 μM after administration of NAC and C_{60}FAS, respectively, during the experiment.

The content of GSH was 5.7 ± 0.6 M during the development of fatigue (1.9 ± 0.2 M in the intact group) and 4.2 ± 0.4 M and 3.8 ± 0.4 after administration of NAC and C_{60}FAS, respectively, during the experiment.

Thus, the above in vivo results demonstrate a positive cumulative effect on the resumption of the contractile function of skeletal muscles with the therapeutic use of biocompatible water-soluble C_{60} fullerenes. This opens up broad prospects for their use in a complex of rehabilitation procedures aimed at restoring motor activity, in clinical and sports medicine, as well as for preliminary therapeutic procedures before work, associated with extreme physical exertion. However, this requires further clinical trials (dose-effect).

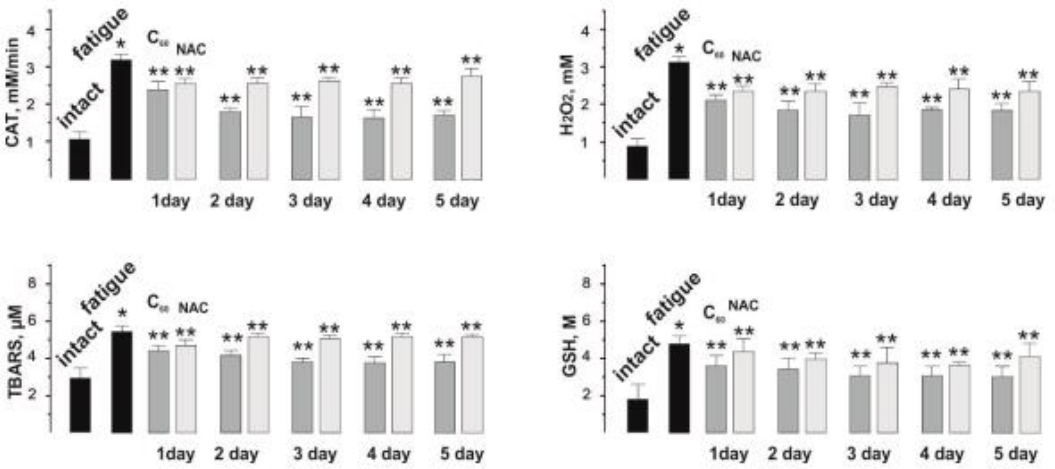

Figure 7. Indicators of pro- and antioxidant balance (CAT, H_2O_2, TBARS, and GSH) in the blood of rats after application of three-component stimulation and administration of C_{60}FAS (C_{60}) and NAC for 1, 2, 3, 4, and 5 days. * $p < 0.05$ relative to intact group; ** $p < 0.05$ relative to the fatigue group (without drugs).

4. Conclusions

Thus, the obtained data indicate that the applied therapeutic drugs have the most significant effects on the 2nd and especially the 3rd pool of skeletal muscle stimulation. The use of C_{60}FAS on the first day by (50–80)% has a stronger effect on the resumption of muscle biomechanics after the beginning of fatigue than NAC, and its five-day use additionally increases the therapeutic effect by (12–15)% for all studied biomechanical markers, except for the time of reaching maximum force response. There is also a positive therapeutic trend towards a decrease in all described biochemical parameters by about (12–15)% after administration of NAC and by (20–25)% after using C_{60}FAS. Five-day use of NAC did not significantly change the studied parameters, while long-term use of C_{60}FAS increased the therapeutic effect by about (7–9)%. This indicates the presence of a more powerful compensatory activation of the endogenous antioxidant system by C_{60} fullerene in response to prolonged muscle stimulation.

In summarizing, C_{60} fullerene can influence the activity of endogenous antioxidants, preventing dysfunction in an active muscle and, thus, maintaining it within the physiological norm throughout the entire process of its contraction. This provides the potential possibility of using C_{60}FAS as a therapeutic agent capable of reducing and correcting the pathological conditions of the muscular system that occur during fatigue processes in the skeletal muscles.

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Institutional Review Board Statement: The study protocol was approved by the Bioethics Committee 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.3446–IV 21.02.2006, Kyiv, on the Protection of Animals from Cruelty during medical and biological research.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

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Effect of C_{60} Fullerene on Recovery of *Muscle Soleus* in Rats after Atrophy Induced by Achillotenotomy

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Abstract: Biomechanical and biochemical changes in the muscle soleus of rats during imitation of hind limbs unuse were studied in the model of the Achilles tendon rupture (Achillotenotomy). Oral administration of water-soluble C_{60} fullerene at a dose of 1 mg/kg was used as a therapeutic agent throughout the experiment. Changes in the force of contraction and the integrated power of the muscle, the time to reach the maximum force response, the mechanics of fatigue processes development, in particular, the transition from dentate to smooth tetanus, as well as the levels of pro- and antioxidant balance in the blood of rats on days 15, 30 and 45 after injury were described. The obtained results indicate a promising prospect for C_{60} fullerene use as a powerful antioxidant for reducing and correcting pathological conditions of the muscular system arising from skeletal muscle atrophy.

Keywords: *muscle soleus* of rat; achillotenotomy; atrophy; C_{60} fullerene; biomechanical and biochemical parameters of skeletal muscle contraction

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1. Introduction

Functional unloading of mammalian skeletal muscles caused by partial immobilization can cause their atrophy. In this case, the deepest atrophic changes are observed in the *muscle soleus*—the key postural muscle [1]. Possessing a pronounced plasticity, the skeletal muscle of mammals is able to rearrange its structural and metabolic profile, depending on the nature of contractile activity and changes in external conditions [2]. Regular strength training significantly increases the intensity of protein synthesis and, as a result, leads to hypertrophy of muscle fibers [3]. Conversely, functional unloading leads to suppression of protein synthesis and activation of proteolysis, which is reflected in a decrease in the diameter of muscle fibers (atrophy) and loss of their strength of contraction [4]. Muscle atrophy caused by prolonged inactivity is associated with both suppression of the intensity of protein synthesis and activation of intracellular proteolysis systems, which has been found in numerous animals and human model studies [5]. It has been shown that even short periods (5 days) of unuse of muscles can cause a significant loss of their mass and strength of contraction as well as accompaniment of physiological molecular rearrangements [6]. Both slow and fast fibers undergo atrophy; the largest fibers in an individual muscle usually show the greatest atrophic response. Atrophy of muscle fibers

plateau after about 14 days of immobilization or gravitational inactivity. The increase in fatigue under these conditions reflects the loss of muscle and fiber mass. The glycolytic capacity of muscles and muscle fibers continues after immobilization for 30–40 days [7].

Since the initial discovery of MuRF1 and MAFbx as two muscle-specific E3 ubiquitin ligases, several additional mediators of muscle atrophy have been discovered, providing new insights into how muscle atrophy occurs at the molecular level [8]. Traditionally, two clinical models have been used to simulate unuse of the hind limbs in rats: Achilles tendon rupture (Achillotenotomy, AT) and suspension of the hind limbs. Significant atrophy of the gastrocnemius muscle occurs in both cases, starting at day 10 of the study. Degradation of the basement membrane of muscle fibers leads to impaired muscle contractility. A significant decrease in the force of contractions of the gastrocnemius muscle, isometric tetanic force and the rate of contraction after tendon rupture has been demonstrated [9]. Achilles tendon rupture and subsequent muscle atrophy leads to functional impairments, which can also be caused by morphological changes in the muscle–tendon block. The functional characteristics of the injured limb will be impaired regardless of the time that has elapsed after the operation, and these impairments occur together with changes in the morphology of the muscle–tendon. Disorders can persist for many years in the postoperative period, although they can be more pronounced with high-speed activity [10] and disrupt nonlinear processes in muscle biomechanics [11].

Selection of AT as a model for imitation of unuse of rats' hind limbs is based on its weightier social significance. Acute rupture of the Achilles tendon is a common injury that can lead to disability. Over the past decade, in the treatment of acute rupture of the Achilles tendon and the resulting atrophy of muscle groups, there has been a transition from surgical treatment to non-surgical treatment. However, the optimal protocol for non-surgical treatment is under development [12]. The problem of increasing the length of the Achilles tendon after its rupture remains unresolved, which is associated with a decrease in the volume of the calf muscles and a persistent deficit in plantar flexion strength after surgical recovery. The deficit in muscle strength and volume is partially compensated by hypertrophy: a deficit in *muscle soleus* volume from 11% to 13% and a deficit in plantar flexion strength from 12% to 18% persist even after long-term follow-up [13]. Muscle atrophy, joint stiffness, osteoarthritis, infection, necrosis and ulceration of the articular cartilage are known complications caused by prolonged immobilization of surgically repaired Achilles tendon ruptures [14].

The main methods of treating such injuries, based on the surgical repair of tendon ruptures, have a number of significant drawbacks [15]. Serious degradation of the muscular apparatus always occurs during therapeutic procedures and the rehabilitation period of recovery [16]. Thus, the development of a rehabilitation protocol is an essential aspect of restoration of the pre-injury activity levels. Despite several available trials, which compare different treatment regimens, there is still no consensus on the optimal protocol [17].

Recently, the use of antioxidant therapy in the early stages of the development of muscle atrophy demonstrates the promise of this approach. The authors of [18] showed that the use of an antioxidant, curcumin, leads to a decrease in oxidative stress and the activity of proteolytic pathways and, as a consequence, decreases the degradation of muscle protein during the development of muscle atrophy. It has been shown that licorice flavonoid oil that contains glabridin, and exhibits strong antioxidant properties, increases muscle mass in mice with muscle atrophy. Oral administration of glabridin prevented induced protein degradation in the tibialis anterior muscle of mice. This indicates that the antioxidant glabridin is an effective food ingredient for preventing skeletal muscle atrophy [19]. At the same time, no advantages of oral administration of the studied additives in collagen synthesis or improvement of the biomechanical properties of atrophied muscles were found after 3 weeks of use while studying the effect of vitamin C on the healing of the Achilles tendon in rats. Therefore, the search for an optimal antioxidant for the treatment of muscular atrophy is still ongoing [20].

It is known that C_{60} fullerene is capable of inactivating methyl, superoxide anion and hydroxyl radicals in in vivo and in vitro systems [21,22]. In our previous studies, it was shown that administration of water-soluble C_{60} fullerenes after initiation of ischemic damage leads to significant positive therapeutic effects [23]. A positive trend has been shown when using them for muscle injury [24], muscle dysfunction associated with pesticide poisoning [25], as well as the development of fatigue processes [26]. All these data stimulated us to test water-soluble C_{60} fullerenes as potential therapeutic agents that reduce pathological effects in the muscular system of rats during the development of AT-associated dystrophy.

2. Materials and Methods

The experiments were performed on male Wistar rats aged 2 months weighing 200 ± 6 g. 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 №3446–IV 21.02.2006, Kyiv, on the Protection of Animals from Cruelty during medical and biological research.

Before the start of the study, rats underwent Achilles tendonomy—a cut of the Achilles tendon. The following groups of animals were studied: intact group of animals (n = 7), groups of animals on days 15, 30 and 45 after AT without administration of water-soluble C_{60} fullerenes (n = 7 in each group) and with administration of water-soluble C_{60} fullerenes (n = 7 in each group). In preparation for the experiment, anesthesia of animals was performed by intraperitoneal administration of nembutal (40 mg/kg). The standard preparation included the cannulation (a. carotis communis sinistra) for pressure measurement and laminectomy at the lumbar spinal cord level. *Muscle soleus* of rat was released from the surrounding tissues. Its tendon was cut across the distal part, which was connected to the force sensors. For modulated stimulation of efferents, the ventral roots were cut at the points of their exit from the spinal cord. Research of muscle contraction dynamics was performed under the conditions of muscle activation using the method of modulated efferent stimulation [27]. Filaments of the cut ventral roots were fixed on the stimulating electrodes and cyclic distribution of the stimulus sequence was performed. Stimulation of efferents was performed by electrical pulses lasting 2 ms, generated by the impulse generator. The control of the external load on the muscle was performed using a system of mechanical stimulators. The change in force was measured using strain gauges.

In the process of analyzing the obtained results, the integrated muscle power (calculated area under the force curve) was used as parameter, which is an indicator of the general performance of the muscle with the applied stimulation pools [28]. The development of muscle contractile activity was assessed by the method of calculating time intervals when 50% of the levels of strength responses were reached during stimulation.

An aqueous colloidal solution of C_{60} fullerenes was obtained using the original ultrasonic technology [29,30]. At a maximum concentration of 0.15 mg/mL, it remains stable for 18 months at a storage temperature of +4 °C.

The data of the authors [31] show that the time before the onset of atrophy caused by muscle unloading is the most optimal for therapeutic intervention in preventing skeletal muscle atrophy, which is associated with the redox balance. Based on this, the protocol of our research assumed the initiation of the administration of water-soluble C_{60} fullerenes immediately after the initiation of the injury.

Water-soluble C_{60} fullerene was administered orally at a dose of 1 mg/kg each day of the experiment. An appropriate amount of the solution was poured into a rat drinker, each of which was kept in a separate cage. Further feeding and watering of the animal was carried out only after the emptying of the drinker.

It is important to note that the selected dose of water-soluble C_{60} fullerene in our experiments is significantly lower than the LD_{50} value, which was 600 mg/kg body weight

when administered orally to rats [32] and 721 mg/kg when administered intraperitoneally to mice [33].

The level of enzymes content in the blood of experimental animals, namely, the thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H_2O_2), reduced glutathione (GSH) and catalase activity (CAT)), as markers of muscle injury, was determined using clinical diagnostic equipment—a haemoanalyzer [25].

Single muscle fibers were isolated surgically using microsurgical instruments under a binocular microscope. In each experiment, we removed the muscle soleus, which was dissected in its thickest part (2/5 of its length from the proximal end). A Nikon inverted microscope (×600) and a Panasonic video camera system were used to determine the diameter of a single *soleus* fiber. Fiber diameter was determined using a calibration eyepiece as the average of three measurements and, accordingly, the cross sectional area (CSA) was calculated.

Each of the experimental curves shown in the figures is the result of averaging 10 similar tests. The same averaging proportions were used in each of the groups of animals studied. Statistical processing of measurement results was performed by methods of variation statistics using software Original 9.4.

Data are expressed as the means ± 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. Dynamics of Muscle Soleus Contraction Force in Rats

On day 15 after AT initiation, the maximal *muscle soleus* contraction force of rats induced by 6 s with nonrelaxation stimulation pools decreased to 58 ± 2% in the first contraction and to 23 ± 5% in the tenth, relative to the intact group. Thus, there was a sharp decrease in muscle strength activity already at the first contractions with a progressive decrease in the studied biomechanical parameters (Figures 1 and 2). On days 30 and 45 after AT, the maximal force response decreased to 79 ± 5% in the first reduction and 59 ± 4% in the tenth and to 88 ± 7% in the first reduction and 78 ± 7% in the tenth, respectively. After using C_{60} fullerene therapy, these values were 65 ± 6% in the first reduction and 45 ± 6% in the tenth, 84 ± 7% in the first reduction and 77 ± 3% in the tenth, 95 ± 9% in the first reduction and 91 ± 5% at the tenth on days 15, 30 and 45 after AT, respectively. The therapeutic effect averaged 45–55%.

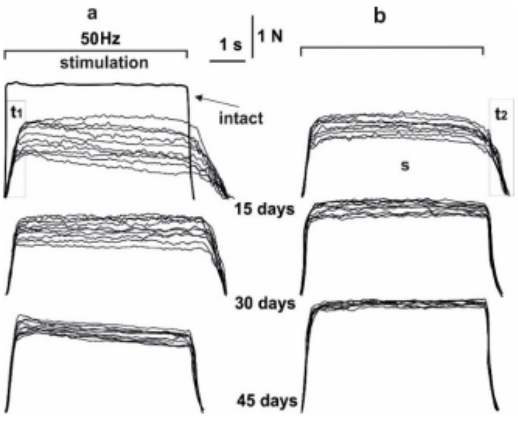

Figure 1. The force of contraction of the *muscle soleus* after AT in rats, caused by 10 consecutive 6 s non-relaxation pools of stimulation: without C_{60} fullerenes administration (**a**); with C_{60} fullerenes administration at a dose of 1 mg/kg (**b**). Native muscle–intact, t_1–time of the maximum strength response development, t_2–recovery time of strength parameters to their initial values, S–integrated power of muscle contraction, calculated as the total area under the corresponding strength curve.

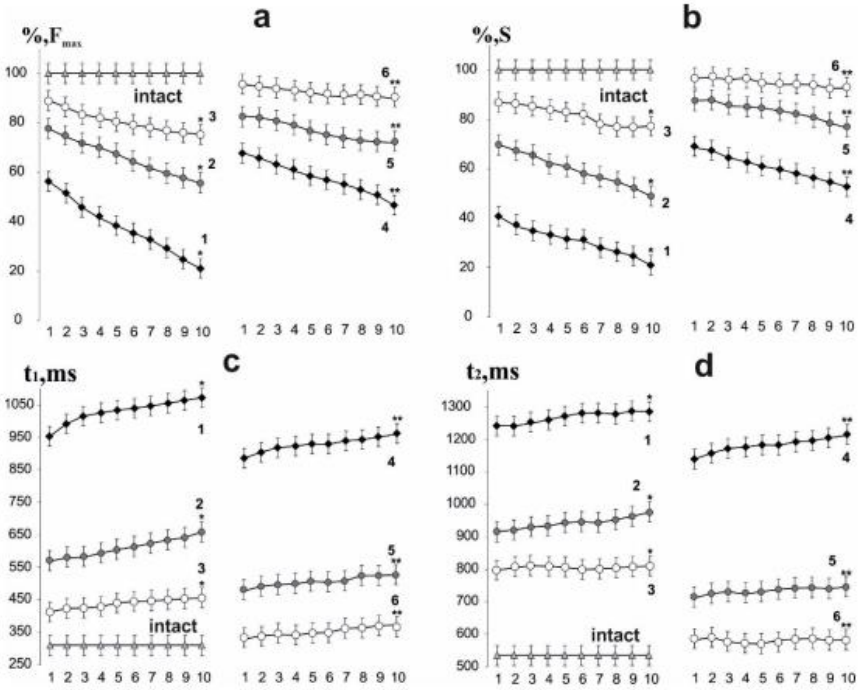

Figure 2. Biomechanical parameters of *muscle soleus* after AT in rats at 10 consecutive 6 s non-relaxation contractions: change in the maximum force response as a percentage of the values in the intact group (**a**); integrated muscle power as a percentage of values in the intact group (**b**); time of development of the maximum force response (**c**); recovery time of force parameters to their original values (**d**). Native muscle–intact; 1,2,3–the values of the corresponding parameters on 15th, 30th and 45th days after AT, respectively, without administration of C60 fullerenes (*$p < 0.05$ compare to the intact group at all 1, 2,…10 consecutive contractions); 4,5,6–the values of the corresponding parameters on 15th, 30th and 45th days after AT, respectively, after using C60 fullerenes at a dose of 1 mg/kg (**$p < 0.05$ compared to the group without the use of C60 fullerene at all 1, 2,…10 consecutive contractions).

The decrease in the integrated power of muscle contraction on the 15th day after initiation of AT was 41 ± 2% after the first contraction and 22 ± 4% after tenth, respectively, relative to the intact group. On days 30 and 45, these indicators were 70 ± 3% and 53 ± 4%, 84 ± 7% and 79 ± 7% after first and tenth contractions, respectively. These indicators were 73 ± 3% and 59 ± 7%, 85 ± 6% and 78 ± 7%, 94 ± 3% and 92 ± 6% after the first and tenth contractions, respectively, using C_{60} fullerene therapy on days 15, 30 and 45 after AT. The therapeutic effect averaged 35–40%.

3.2. Estimation of the Time to Reach the Maximum Force Response and Recovery of Muscle Soleus Force Parameters in Rats

The time to reach the maximum force response is one of the most important biomechanical parameters, since its change significantly affects the quality of targeted movements and the adequate implementation of motoneuronal pools. On the 15th day after AT activation, an increase in this indicator was recorded from 961 ± 5 ms after the first contraction to 1070 ± 7 ms after the tenth, in comparison with the intact group (275 ± 9 ms). On days 30 and 45 after AT activation, these indicators were 570 ± 11 and 660 ± 14 ms, 400 ± 7 and 445 ± 7 ms after the first and tenth contractions, respectively. After using C_{60} fullerene therapy, a correction of these parameters was recorded: 872 ± 12 and 954 ± 8 ms, 460 ± 13 and 524 ± 12 ms, 336 ± 14 and 378 ± 12 ms after the first and tenth contractions on days 15, 30 and 45, respectively. The therapeutic effect averaged 50–60% on the 15th day after AT and 20–25% after 45 days. This can be explained by the fact that pathological factors

affecting the time to reach the maximum force response are on the first days after AT and decrease their pathological effect with an increase in the time after the described injury. The progressive decrease in the force response lasts at least 15 days, after which the recovery process takes place [34].

The recovery time of force parameters to their initial values is directly affected by an increase in muscle stiffness and a change in the elastic properties of tendon components. On the 15th day after AT activation, its increase was recorded as 1240 ± 58 ms after the first contraction and 1290 ± 15 ms after the tenth in comparison with intact group (521 ± 16 ms). On days 30 and 45, these indicators were 900 ± 16 ms and 993 ± 21 ms, 790 ± 17 and 800 ± 18 ms after the first and tenth contractions, respectively. Its slight growth with an increase in the number of 6 s non-relaxation contractions against its significant decrease with increasing of time after AT should be noted. With the use of C_{60} fullerene therapy, a significant decrease in the recovery time of force parameters to the initial values was recorded: 1123 ± 19 and 1211 ± 15 ms, 722 ± 18 and 749 ± 13 ms, 590 ± 24 and 593 ± 19 ms after the first and tenth reduction on the 15, 30 and 45 days after AT, respectively. Thus, the obtained data indicate a positive dynamic of the therapeutic use of water-soluble C_{60} fullerenes in a daily dose of 1 mg/kg, which leads to a decrease in the level of muscle damage severity by an average of 25–35%.

3.3. Analysis of Fatigue Processes in the Muscle Soleus of Rats after AT Using 1 Hz Stimulation

Previously, an increase in the amount of intramuscular connective tissue due to trauma was revealed, which, apparently, occurs simultaneously with muscle atrophy and loss of muscle capillarity [35]. These factors are key to the onset of increased muscle fatigue in the active muscle. Therefore, the next stage of our research was to analyze the occurrence of the fatigue processes in the *muscle soleus* after AT upon application of stimulation. Registration of the contraction force with the use of 1 Hz stimulation for 1800 s showed a decrease in the integrated muscle power (Figure 3): it was 28 ± 2%, 59 ± 6% and 64 ± 4% relative to the intact group on days 15, 30 and 45 of the experiment, respectively. The use of water-soluble C_{60} fullerenes improved this indicator to 61 ± 2%, 78 ± 4% and 88 ± 7% on days 15, 30 and 45 of the experiment, respectively. The therapeutic effect was more than 50%, which may be due to the antioxidant properties of C_{60} fullerenes to correct fatigue processes in the active muscle [36].

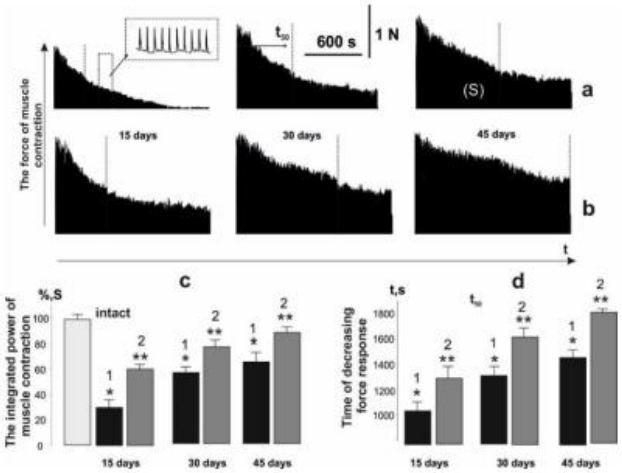

Figure 3. Biomechanical parameters of *muscle soleus* after AT in rat at 1 Hz stimulation for 1800 s: without C_{60} fullerenes administration (**a**); with the use of C_{60} fullerenes at a dose of 1 mg/kg (**b**); integrated muscle power (S), presented as a percentage of values in the intact group (**c**); time reduction of the force response by 50% from the initial values (t_{50}) (**d**). Native muscle–intact; 1,2–the

corresponding values of the parameters without and with C_{60} fullerenes use, respectively. *$p < 0.05$ compare to the intact group; **$p < 0.05$ compare to the group without the use of C_{60} fullerene.

The time for force response to decrease by 50% of the initial values (t_{50}) without C_{60} fullerene therapy was 1020 ± 42, 1310 ± 65 and 1490 ± 85 ms on days 15, 30 and 45 of the experiment, respectively. After using of water-soluble C_{60} fullerenes this indicator was 1325 ± 72, 1680 ± 77, and 1780 ± 59 ms, respectively, which shows its 50% therapeutic effect at the stages of maintaining the maximum force responses during the development of fatigue processes.

3.4. Analysis of the Occurrence of Smooth Tetanic Contraction of Muscle Soleus in Rats

The most important quantitative indicator of skeletal muscles work in the process of functioning is the rate of smooth tetanic contraction occurrence. Even minimal physiological or biochemical destructive changes in the structure of myocytes and motoneuronal pools, changes in muscle stiffness and electrical properties of membranes or the duration of hyperpolarization significantly change the time of smooth tetanic contractions occurrence [37]. Moreover, during muscle activity, its individual motor units generate unfused tetanic contractions, which are characterized by variable strength and varying degrees of fusion. The synchronization of this process depends on many factors and is a vulnerable element in the development of pathological processes in the muscle [38]. Therefore, the next step was to study biomechanical markers of the appearance of smooth tetanic contractions.

Using of stimulation pools with increasing frequency (Figure 4), the smooth tetanic contractions (maximum force response) appeared after 3450 ± 12 ms and reached 97 ± 8 mN (Figure 5). *Muscle soleus* after AT did not reach the stage of smooth tetanic contraction throughout the experiment. The maximum force of a single contraction (f_{max}) was 43 ± 2, 67 ± 4, and 87 ± 2 mN on days 15, 30 and 45 of the experiment, respectively. The use of water-soluble C_{60} fullerenes increased these indicators to 72 ± 3, 79 ± 5, and 94 ± 2 mN on days 15, 30 and 45 of the experiment, respectively. The minimum value of the force response in one tooth of dentate tetanus (f_{min}) slightly decreased to 22 ± 3, 17 ± 2 and 5 ± 1 mN on days 15, 30 and 45 of the experiment, respectively. It should be noted that a decrease in this parameter to zero leads to the appearance of smooth tetanus. The use of C_{60} fullerene changed the biomechanical parameters of the transition of *muscle soleus* from dentate to smooth tetanus, which appeared 4350 ± 32 and 3650 ± 32 ms on days 30 and 45 after AT, respectively. All the described biomechanical parameters after the application of C_{60} fullerene showed positive therapeutic dynamics at the level of 23–29%.

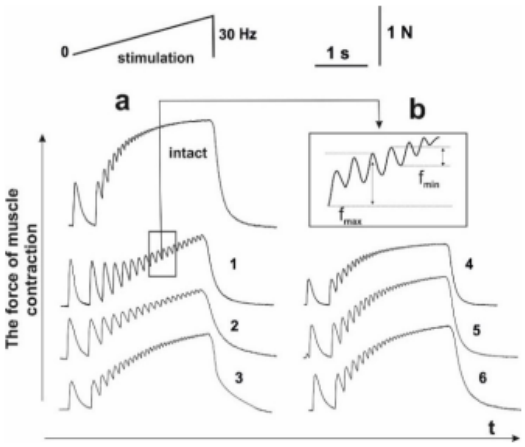

Figure 4. Mechanograms of the transition of *muscle soleus* after AT in rats from dentate to smooth tetanus with the use of increasing stimulation with a maximum frequency of 30 Hz for 6 s: without C_{60} fullerenes administration (**a**); with C_{60} fullerenes administration in a daily dose of 1 mg/kg (**b**). Native muscle–intact; f_{max} is the maximum force of a single contraction, f_{min} is the minimum value

of the force response in one tooth of the dentate tetanus; 1,2,3–the values of the corresponding parameters on 15, 30 and 45 days after AT, respectively, without C_{60} fullerenes administration; 4,5,6–the values of the corresponding parameters on 15, 30 and 45 days after AT, respectively, with the use of C_{60} fullerenes at a dose of 1 mg/kg.

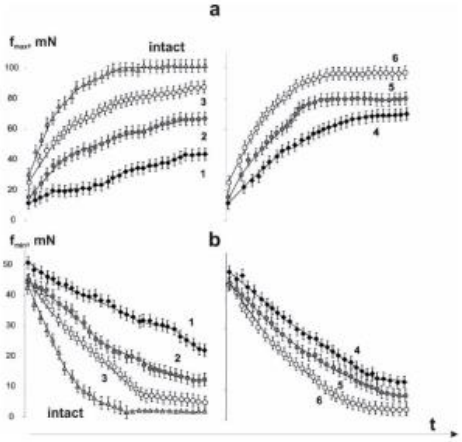

Figure 5. Changes in f_{max} (**a**) and f_{min} (**b**) parameters of *muscle soleus* after AT for each of the single contractions during the transition of the force response to smooth tetanus using an increasing stimulation signal with a maximum frequency of 30 Hz for 6 s: 1,2,3–parameter values on days 15, 20 and 45 after AT, respectively, without C_{60} fullerenes administration; 4,5,6–the values of the parameters on days 15, 20 and 45 after AT, respectively, with the use of C_{60} fullerenes at a dose of 1 mg/kg.

3.5. Changes in the Body Weights of Animals and the Muscle Soleus, the Value of the Maximum Strength of a Single Tetanic Contraction of an Isolated Muscle, Normalized to the Value of CSA, after AT

The weight of rats of all groups slightly increased during the experiment; this change was taken into consideration for further calculations (Table 1). The mass of *muscle soleus* normalized to the body weight significantly decreased to 0.27 ± 0.032 g on the 15th day after AT and increased to 0.34 ± 0.018 g on the 45th day (in comparison with intact group, this value was 0.49 ± 0.011 g). In the groups that received C_{60} fullerene, these indicators were 0.32 ± 0.015, 0.35 ± 0.023 and 0.39 ± 0.054 g on days 15, 20 and 45 after AT, respectively, which is on average 35–37% higher than in the previous group.

Table 1. Changes in the weight of the animals' bodies and *muscle soleus*, the values of the maximum strength of a single tetanic contraction of an isolated muscle (P_0) and P_0/CSA on days 15, 30 and 45 after AT.

Group	Rat Weight, g	Soleus Weight, mg	Soleus Weight / Rat weight	P_0, mN	P_0/CSA, N/cm²
intact	205 ± 8	102.4 ± 1.5	0.49 ± 0.011	882.4 ± 14.3	23.4 ± 1.2
15 days	231 ± 6 *	63.4 ± 1.8 *	0.27 ± 0.032 *	432.5 ± 16.1 *	14.4 ± 2.5 *
30 days	243 ± 4 *	73.4 ± 1.2 *	0.30 ± 0.015 *	676.5 ± 11.6 *	17.6 ± 7.3 *
45 days	250 ± 6 *	86.4 ± 1.5 *	0.34 ± 0.018 *	693.3 ± 14.1 *	18.6 ± 4.4 *
15 days + C_{60} fullerene	244 ± 5 **	79.4 ± 1.2 **	0.32 ± 0.015 **	602.5 ± 12.2 **	18.1 ± 1.2 **
30 days + C_{60} fullerene	254 ± 2 **	89.4 ± 1.3 **	0.35 ± 0.023 **	711.5 ± 22.5 **	19.2 ± 1.1 **
45 days + C_{60} fullerene	269 ± 7 **	105.4 ± 1.9 **	0.39 ± 0.054 **	782.5 ± 16.3 **	20.3 ± 1.2 **

* $p < 0.05$ compare to intact group; ** $p < 0.05$ compare to the group without C_{60} fullerene administration.

The maximum strength of a single tetanic contraction (P_0) (this value in the intact group was 882.4 ± 14.3 mN) decreased to 432.5 ± 16.1, 676.5 ± 11.6 and 693.3 ± 14.1 mN on days 15, 30 and 45 after AT, respectively. The use of C_{60} fullerene improved this indicator to 602.5 ± 12.2, 711.5 ± 22.5 and 782.5 ± 16.3 mN on days 15, 30 and 45 after AT, respectively, which showed an increase in the P_0 value by more than 30%. The most significant results were shown by changes in the maximum strength of a single tetanic contraction (P_0), normalized to the value of CSA. The decrease in P_0/CSA value to 14.4 ± 2.5, 17.6 ± 7.3 and 18.6 ± 4.4 N/cm² on days 15, 30 and 45 after AT was 61.5, 75.2 and 78%, respectively, in comparison with the value in the intact group (23.4 ± 1.2 N/cm²). With the use of C_{60} fullerene, these indicators were 18.1 ± 1.2, 19.2 ± 1.1 and 20.3 ± 1.2 N/cm² on 15, 30 and 45 days after AT, respectively, which is more than 40% higher than in the previous group. According to the obtained data, it can be concluded that C_{60} fullerenes administration in a daily dose of 1 mg/kg reduces the level of destruction of muscle tissue by 30–35%.

3.6. Analysis of Blood Biochemical Parameters in Rats as Markers of Muscle Injury

Unused muscles atrophy is part of numerous pathologies in which the loss of muscle mass ultimately leads to the depletion of the organism (cachexia). Whether it is caused by muscle failure or disease, muscle loss results in weakness and metabolic co-morbidity. Reactive oxygen species (ROS) are important regulators of cellular signaling pathways that can accelerate proteolysis and suppress protein synthesis [39]. The authors of [40] showed that increased production of ROS in skeletal muscles significantly contributes to their atrophy caused by inactivity. Inflammatory cascade processes that occur immediately after AT are a source of ROS and contribute to the intensification of lipid peroxidation (LPO) processes. As a result of biochemical tests, we determined the levels of LPO secondary products and antioxidants in the blood of rats after AT. The obtained data clearly demonstrate an increased level of markers of peroxidation and oxidative stress (CAT, H_2O_2, TBARS and GSH) after AT and their decrease after C_{60} fullerene therapy (Figure 6).

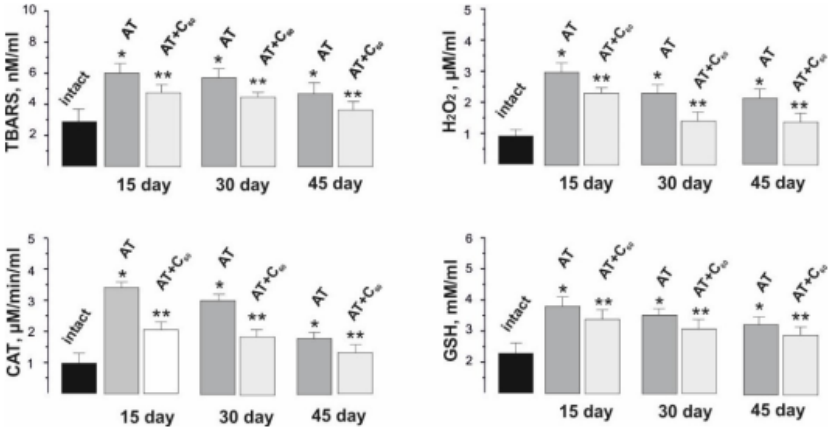

Figure 6. Indicators of pro- and antioxidant balance (TBARS, H_2O_2, CAT and GSH) in the blood of rats after 1 Hz stimulation of *muscle soleus* for 1800 s on 15, 20 and 45 days after AT. * $p < 0.05$ compare to the intact group; ** $p < 0.05$ compare to the group without C_{60} fullerenes administration.

Thus, the CAT level increased from 0.9 ± 0.1 μM/min/mL (in the intact group) to 3.5 ± 0.3, 3.1 ± 0.4 and 1.8 ± 0.6 μM/min/mL on days 15, 30 and 45 after AT, respectively, and decreased to 2.0 ± 0.4, 1.8 ± 0.1 and 1.3 ± 0.5 μM/min/mL on days 15, 30 and 45 after AT with the use of C_{60} fullerene therapy, respectively. The level of H_2O_2 was 2.8 ± 0.3, 2.5 ± 0.3, and 2.3 ± 0.6 μM/mL on days 15, 30 and 45 after AT, respectively (in the intact group, this value was 0.8 ± 0.2 μM/mL), and decreased to 2.1 ± 0.7, 1.5 ± 0.2 and 1.4 ± 0.6 μM/mL

on days 15, 30 and 45 with the use of C_{60} fullerene therapy, respectively. The TBARS level was 6.1 ± 0.1, 5.5 ± 0.8 and 4.3 ± 0.3 nM/mL on days 15, 30 and 45 after AT, respectively (in the intact group, this value was 2.9 ± 0.2 nM/mL), and 4.3 ± 0.4, 4.1 ± 0.8 and 3.8 ± 0.6 nM/mL on days 15, 30 and 45 after AT with the use of C_{60} fullerene therapy, respectively. The GSH concentration was 3.8 ± 0.4, 3.4 ± 0.2 and 3.2 ± 0.3 mM/mL on days 15, 30, and 45 after AT, respectively (in the intact group, this value was 2,0 ± 0.3 mM/mL), and 3.3 ± 0.2, 3.1 ± 0.7 and 2.9 ± 0.3 mM/mL on days 15, 30 and 45 after AT with the use of C_{60} fullerene therapy, respectively.

Thus, there is a positive change in the described biochemical parameters by approximately 27–30% after therapeutic administration of C_{60} fullerene. This indicates the presence of compensatory activation by C_{60} fullerene of the endogenous antioxidant system in the process of dystrophic changes in the *muscle soleus* caused by AT. In our opinion, C_{60} fullerene can affect the activity of endogenous antioxidants, suppressing the occurrence of destruction in the muscle and, thus, reducing its degradation. The therapeutic effect of water-soluble C_{60} fullerenes on the restoration of tendon structures is also possible, this was confirmed by the previously obtained data about their protective effect in inflammatory and pathological processes in the body [41–43].

Despite the fact that atrophy that occurs after traumatic joint injury has morpho-functional differences against muscle atrophy that develops as a result of their unuse, the main mechanisms leading to changes in muscle mass in this pathology do not differ significantly [44]. Therefore, it can be assumed that there is not a significant difference in the treatment of atrophic pathologies caused by these factors.

4. Conclusions

Based on the obtained data, we can conclude that the positive therapeutic changes in the studied biomechanical and biochemical markers confirm the possibility of using water-soluble C_{60} fullerene (oral administration at a dose of 1 mg/kg each day of the experiment) as a promising nanoagent that can reduce and correct pathological states of the muscular system arising from skeletal muscle atrophy due to unuse.

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Research article

C_{60} fullerene attenuates muscle force reduction in a rat during fatigue development

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ABSTRACT

C_{60} fullerene (C_{60}) as a nanocarbon particle, compatible with biological structures, capable of penetrating through cell membranes and effectively scavenging free radicals, is widely used in biomedicine. A protective effect of C_{60} on the biomechanics of fast (*m. gastrocnemius*) and slow (*m. soleus*) muscle contraction in rats and the pro- and antioxidant balance of muscle tissue during the development of muscle fatigue was studied compared to the same effect of the known antioxidant N-acetylcysteine (NAC). C_{60} and NAC were administered intraperitoneally at doses of 1 and 150 mg kg^{-1}, respectively, daily for 5 days and 1 h before the start of the experiment. The following quantitative markers of muscle fatigue were used: the force of muscle contraction, the level of accumulation of secondary products of lipid peroxidation (TBARS) and the oxygen metabolite H_2O_2, the activity of first-line antioxidant defense enzymes (superoxide dismutase (SOD) and catalase (CAT)), and the condition of the glutathione system (reduced glutathione (GSH) content and the activity of the glutathione peroxidase (GP_x) enzyme). The analysis of the muscle contraction force dynamics in rats against the background of induced muscle fatigue showed, that the effect of C_{60}, 1 h after drug administration, was (15–17)% more effective on fast muscles than on slow muscles. A further slight increase in the effect of C_{60} was revealed after 2 h of drug injection, (7–9)% in the case of *m. gastrocnemius* and (5–6)% in the case of *m. soleus*. An increase in the effect of using C_{60} occurred within 4 days (the difference between 4 and 5 days did not exceed (3–5)%) and exceeded the effect of NAC by (32–34)%. The analysis of biochemical parameters in rat muscle tissues showed that long-term application of C_{60} contributed to their decrease by (10–30)% and (5–20)% in fast and slow muscles, respectively, on the 5th day of the experiment. At the same time, the protective effect of C_{60} was higher compared to NAC by (28–44)%. The obtained results indicate the prospect of using C_{60} as a potential protective nano agent to improve the efficiency of skeletal muscle function by modifying the reactive oxygen species-dependent mechanisms that play an important role in the processes of muscle fatigue development.

1. Introduction

Today, the term "muscle fatigue" refers to a wide range of dysfunctions, namely: physiological, neurological, and psychiatric [1, 2]. Therefore, there are diverse concepts about ways of muscle fatigue development [3], the existence of which is because there is no separate mechanism for the complex process of fatigue development. However, it involves a whole complex of mechanisms of central nervous system dysfunction, peripheral nerve dysfunctions, and muscles themselves. All mechanisms are united by the result of functional changes caused by them, mostly by the impossibility of maintaining the required level of effort by the muscle during contraction [4]. Muscle fatigue is a protective

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mechanism of the body against overloads in general and further development of pain sensitivity of muscles [5]. Its nature and optimal degree are key factors for forming adaptation and increasing the level of functional and physical capabilities of the body. It has been shown that during intense physical load, the duration of recovery periods (active rest) is quite important for maintaining optimal performance and a normal physiological state of actively contracting muscles [6].

During the development of fatigue, there is a slowdown in force generation and muscle relaxation [7]. Skeletal muscle fibers continuously generate reactive oxygen species (ROS) at a slow rate, which increases during muscle contraction. This activity-dependent increase in ROS production contributes to skeletal muscle fatigue during strenuous exercise. Experimental evidence suggests that ROS of muscle origin primarily act on myofibrillar proteins, suppressing calcium sensitivity and reducing contraction force [8]. More intensive exposure to ROS leads to losses in calcium regulation, which mimic pathological changes and are irreversible.

Oxidative stress is a generally recognized pathophysiological factor in the formation of muscle fatigue and overexertion under conditions of excessive physical activity [9]. There are several sources of ROS production in skeletal muscles that are activated during muscle contraction: mitochondrial respiratory chain, lipoxygenase pathway of arachidonic acid metabolism, NADPH-oxidase, and xanthine oxidase [10, 11]. Superoxide, hydroxyl radicals, and hydrogen peroxide are considered to be the most reactive oxygen derivatives that can be formed during the development of muscle fatigue in enzymatic and non-enzymatic reactions [12]. It is known that pathological effects in muscle tissue arise due to excessive accumulation of ROS, peroxides, and their secondary products with the inability of the antioxidant system to ensure the maintenance of prooxidant-antioxidant balance [13]. The functional basis of the antioxidant defense system is formed by the glutathione system, the components of which are glutathione and enzymes that catalyze the reactions of reverse transformation (oxidation or recovery) [14].

During the intensive physical activity of skeletal muscle, lipid peroxidation (LPO) is the primary reaction in the chain of physicochemical transformations that lead to the destruction of the lipoprotein complex of myocyte membranes, disruption of their transport functions, inhibition of oxidative phosphorylation processes, and energy generation, which ultimately reduces cell viability and contributes to muscle fatigue [15]. However, the dynamics of lipoperoxidation product accumulation and the duration of such changes depend on many factors, one of which is the type of muscle fibers, because the latter are characterized by specific metabolic reactions and antioxidant protection features [12]. It is known that there are two types of fibers in the muscle structure: slow and fast-contracting ones. These fibers are different independent functional units distinguished not only by contractile but also by morphological and biochemical properties [16].

Biocompatible carbon nanostructures can be considered potential antioxidants to affect the muscular system, some of which are C_{60} fullerenes [17, 18]. C_{60} molecule is a rather powerful electron acceptor, capable of adding up to six electrons. It is the double chemical bonds on the almost spherical surface of C_{60} fullerene that are electron-deficient ones, that determine its ability to effectively capture free radicals [19, 20]. Earlier in *in vivo* experiments, we tested the antioxidant properties of C_{60} fullerenes about the development of some muscle pathologies [21]. Thus, we can assume the correction of muscle fatigue processes due to the protective effect of C_{60} fullerene, as a powerful antioxidant, on the contractile muscular apparatus.

Here, we studied the antioxidant effect of C_{60} fullerene on the biomechanics of fast (*m. gastrocnemius*) and slow (*m. soleus*) muscle contraction in rats, the pro- and antioxidant balance of their body during the development of muscle fatigue compared to the effect of the known antioxidant N-acetylcysteine (NAC) [22].

2. Materials and methods

2.1. Preparation and characterization of C_{60}FAS

To obtain C_{60} fullerene aqueous solution (C_{60}FAS), a method based on the transfer of C_{60} molecules (Sigma Cat. No. 379646) from toluene into the water followed by ultrasound treatment was applied [23, 24]. The mechanism of C_{60} molecule dispersal in an aqueous solution could be explained by a formation of a covalent bond between hydroxyls and carbons in the C_{60} fullerene cage as a result of ultrasound treatment, which culminates in consequent easy C_{60} molecule dissolution [25]. The obtained C_{60}FAS at a maximum concentration of 0.15 mg mL^{-1} was stable for 18 months at +4 °C.

The structural state of C_{60}FAS was studied by the atomic force microscopy (AFM) technique [26]. To do this, a drop of C_{60}FAS was applied to the atomically smooth surface of the substrate, and the measurements were carried out after the complete evaporation of water. Freshly cleaved mica surface (muscovite, grade V1) was used as a substrate for AFM research. Measurements were carried out on the system "Solver Pro M" (NT-MDT, Russia) in tapping mode using AFM probes RTESPA-150 (Bruker, USA).

2.2. Animals

Male Wistar rats (170 ± 12 g, 2-month-old) were bred and housed in standard temperature conditions (21–23 °C), lighting (12/12 h light-dark cycle), at humidity (30–35%). All animals had unlimited access to chow and tap water. The study was carried out in strict accordance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986) and was approved by the Bioethical Committee of the ESC "Institute of Biology and Medicine" of the Taras Shevchenko National University of Kyiv, Ukraine (ethic code: No. 3447–IV 21.02.2006).

The following groups of animals were tested: experimental groups - after 1,2,3,4 and 5 days of C_{60} fullerene ($n = 7$) and NAC ($n = 7$) administration, respectively, which were compared with the control ("fatigue", saline administration) ($n = 7$) and intact ("norm", no fatigue) ($n = 7$) groups.

The research protocol involved intraperitoneal injection of C_{60} fullerene and NAC at a daily dose of 1 and 150 mg kg^{-1}, respectively, 1 h before the experiment for 5 days. The choice of the most optimal dose of the applied drugs, showing a positive protective effect on the development of fatigue processes in the skeletal muscle, is due to the results of our previous studies [27, 40]. An increase/decrease in the range of tested doses did not reveal significant changes in muscle dynamics and, in our opinion, the doses used are optimal.

It is important to note that the selected dose of water-soluble C_{60} fullerene in our experiments is significantly lower than the LD_{50} value, which was 600 mg kg^{-1} body weight when administered orally to rats [19] and 721 mg kg^{-1} when administered intraperitoneally to mice [28].

2.3. Biomechanical analysis

The object of the study was the slow muscle, *m. soleus*, and fast muscle, *m. gastrocnemius*.

Animals under deep anesthesia (ketamine (100 mg kg^{-1}, Pfizer, USA) combined with xylazine (10 mg kg^{-1}, Interchemie, Holland)) underwent a tracheotomy and were connected to an artificial lung ventilator. Then the corresponding muscles were dissected, carefully isolating them from the surrounding tissues. The animal was fixed in a stereotaxic machine with a system of rigid fixation of the head, pelvis, and limbs. The isolated nerve was fixed on a bipolar platinum wire electrode for further electrical stimulation. The edges of the skin on the hind limbs around the incision were sutured to the armature of the machine, and the formed trays with

muscle and nerve were filled with vaseline oil. During the operation and the experiment itself, the heart rate was monitored. If necessary, a mixture of physiological saline, rheopolyglucin, and glucose was administered, and anesthesia was continued by intraperitoneal administration of a mixture of ketamine/xylazine (¼ of the initial dose) every 30–40 min until the end of the experiment. Body and oil temperatures were maintained at 37–38 °C with an infrared lamp.

The muscle was connected via the Achilles tendon to a servo-control muscle puller. A linear motor under position servo-control was used as a muscle puller. The muscle tension was measured by semi-conductor strain gauge resistors glued on a stiff steel beam mounted on the moving part of a linear motor. The puller's stiffness exceeded 0.06 N mm^{-1}, while the time constants of the length transients did not exceed 60 ms.

Muscle fatigue was induced by successive stimulation impulses with a frequency of 50 Hz and a duration of 5 s each, without a relaxation period between them. The sum of such stimulation signals was 500 s, followed by 5 min of relaxation. The number of stimulation pools was three. Each series of stimulation consisted of separate series of rectangular 2 ms impulses. The current strength at which the muscle began to contract was considered the threshold, further stimulation was performed with a force of 1.3–1.4 of the threshold [29].

During the analysis of the results, we used quantitative parameters – integrated muscle power (calculated area under the strength curve), which is an indicator of its general performance under the applied stimulation pools [30].

2.4. Biochemical analysis

LPO was measured from the formation of thiobarbituric acid-reactive substances (TBARS) using the method [31]. TBARS were isolated by boiling tissue homogenates for 15 min at 100 °C with a thiobarbituric acid reagent and measuring the absorbance at 532 nm. The results were expressed as nM mg^{-1} of protein using $\epsilon = 1.56 \times 10^5$ $mmol^{-1}$ cm^{-1}.

The H_2O_2 concentration in the tissue homogenates was measured using the method, which is based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange (o-cresolsulphonephthalein 3′,3″-bis[methylimino] diacetic acid, sodium salt). This method is extremely sensitive and is used to measure low levels of water-soluble hydroperoxide present in the aqueous phase. To determine the H_2O_2 concentration, 500 μL of the incubation medium was added to 500 μL of an assay reagent. The absorbance of the Fe^{3+}-xylenol orange complex (A560) was detected after 45 min. Standard curves of H_2O_2 were obtained for each independent experiment by adding variable amounts of H_2O_2 to 500 μL of basal medium mixed with 500 μL of an assay reagent. Data were normalized and expressed as μM H_2O_2 per mg protein [32].

Measurements of TBARS adducts provide unambiguous evidence for LPO, and an increase in the abundance of these adducts is likely to be reflective of increased oxidative stress. However, these assays are unspecific since TBA generates chromogens from many biomolecules other than MDA, making quantification of the total extent of LPO somewhat challenging [33]. Therefore, to obtain a complete picture of the dynamics of the oxidative processes, we additionally measured select robust indices to assess the ratio of ROS generation/removal such as the activity of antiradical and anti-peroxide enzymes (superoxide dismutase (SOD), catalase (CAT), selenium-dependent glutathione peroxidase (GP_x)) and reduced glutathione (GSH) level.

Total SOD activity was measured by the method [34], which is based on the inhibition of autooxidation of adrenaline to adrenochrome by SOD contained in the examined samples. The results were expressed as specific activity of the enzyme in units per mg protein. One unit of SOD activity is defined as the amount of protein, causing 50% inhibition of the conversion rate of adrenaline to adrenochrome, under specified conditions.

CAT activity was measured by the decomposition of hydrogen peroxide, determined by a decrease in the absorbance at 240 nm [35].

A GP_x activity was determined according to the method [36]. The rate of NADPH oxidation followed at 340 nm.

The GSH level was determined as described in [37]. The tissue sample was mixed with sulphosalicylic acid (4%) and incubated at 4 °C for 30 min. Thereafter, it was centrifuged at 1200 × g for 15 min at 4 °C and 0.1 mL of this supernatant was added to phosphate buffer. The yellow color developed was read immediately at 412 nm. The GSH content was calculated as mmol L^{-1} mg^{-1} protein ($\varepsilon_{412} = 13.6 \times 10^3$ mol^{-1} cm^{-1}).

2.5. Statistical analysis

Statistical processing of the measurement results was performed by methods of variational statistics using the software Origin 9.4. Each of the experimental force curves obtained in the work is the result of averaging 10 similar experiments. At least three repetitions were performed for each biochemical measurement. Data are expressed as the means ± 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. AFM analysis of C_{60}FAS

The biological activity of pristine C_{60} fullerene largely depends on its concentration in the aqueous medium, and the size distribution of the formed nanoparticles, which, in particular, explains some inconsistencies in the toxicity of C_{60} fullerene [38, 39].

Clear AFM images of C_{60} fullerene were obtained (Figure 1a), which indicates the high chemical purity of both the material and the solvent, and the measurement conditions. The images show chaotically placed objects, which in shape and size can be divided into two groups. The first of them includes point objects up to 10 nm high, among which the maximum number was with heights in the range of 0.7–3 nm (Figure 1b). The height of the smallest of them (0.7 ± 0.2 nm) agrees well with the molecular diameter of C_{60}, which allowed us to identify them as individual molecules. Larger objects correspond to C_{60} fullerene bulk clusters. They had symmetrical bell-shaped profiles with sharp maxima in the middle. This is a sign that the aggregation of some molecules into clusters took place in C_{60}FAS before they were applied to the substrate.

The second group includes objects with a height of 10–100 nm of irregular shape (Figure 1c and selected fragments in Figure 1a). They are characterized by fuzzy contours or the presence of protrusions with a thickness of one monolayer at the edges. This is characteristic of the self-assembly of molecules on the substrate surface during deposition from solution to form bulk aggregates according to the known Volmer – Weber islet growth mechanism.

3.2. Analysis of muscle contraction force

Recording the contractions force of *m. soleus* and *m. gastrocnemius* by stimulation pools (Figure 2a and 2b) has revealed a significant difference in the development of their fatigue processes and was 31 ± 3%, 39 ± 1%, and 47 ± 4% at 1,2 and 3 stimulation pools, respectively. These results confirm the data [1] about the greater sensitivity of fast muscle fibers to the development of fatigue.

Mechanograms obtained after 1 h of C_{60} fullerene administration and NAC (Figure 3a) demonstrated a more pronounced effect of C_{60} fullerene, being 19 ± 1%, 17 ± 1%, and 24 ± 3% greater than for NAC in *m. soleus* and 24 ± 2%, 26 ± 1%, and 29 ± 3% greater than in *m. gastrocnemius* at 1,2 and 3 stimulation pools, respectively.

The analysis of mechanograms obtained 2 h after injections of the drugs (Figure 3b) revealed a further slight increase in the effect of C_{60} fullerene, namely by (7–9) ± 1% in *m. gastrocnemius* and (5–6) ± 1% in *m. soleus* at three studied stimulation pools. Note that the effect of NAC did

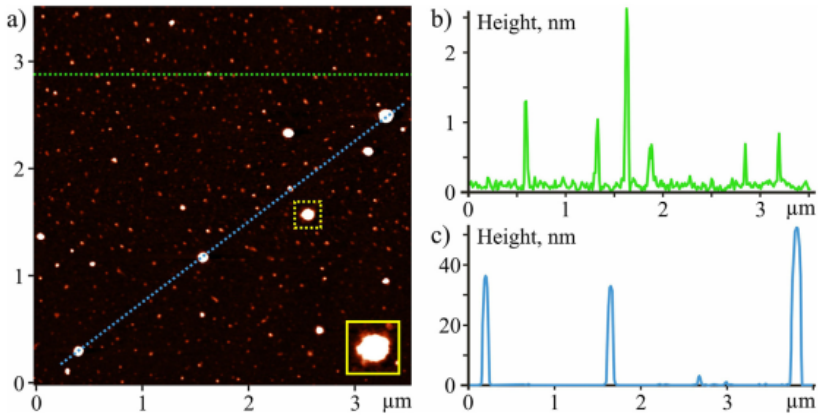

Figure 1. AFM image of the C_{60} fullerene layer deposited from C_{60}FAS (0.15 mg mL^{-1}) on the surface of mica (a), and its Z-section along the lines indicated in the images (b and c).

not change significantly. On this basis, in the future, we analyzed the long-term use of the studied drugs 2 h after their administration.

The analysis of the values of the integrated muscle power 2 h after drug administration on the 1st, 2nd, 3rd, 4th, and 5th days showed an increase in the effect of C_{60} fullerene on the 4th day. The growth of positive effect was 8 ± 1%, 6 ± 1%, and 4 ± 1% on the 5th day in *m. soleus* and 14 ± 1%, 12 ± 1% and 6 ± 1% in *m. gastrocnemius* at 1,2 and 3 stimulation pools, respectively. It should also be noted that the use of NAC did not reveal a significant increase in efficacy already on the 2nd day of its use.

Thus, the most significant effects were observed on the 3rd day of drug use. However, further application of NAC did not cause significant changes in muscle fatigue processes. An increase in the protective effect of C_{60} occurred within 4 days (the difference between 4 and 5 days did not exceed (3–5)%) and exceeded the effect of NAC by (32–34)%.

In summary, the data obtained for the force response of the muscle against the background of the development of muscle fatigue indicate that the administration of C_{60} fullerenes (for at least 4 days) reduces the severity of pathological processes by (35–45)% in slow muscle and by (60–65)% in fast muscle. This confirms that the use of C_{60}FAC in a low dose leads to a decrease in the recovery time of muscle contraction force and an increase in the time of its function [27, 40].

3.3. Analysis of biochemical parameters in muscle tissues

It was found that long-term administration (for 5 days) of C_{60} fullerene led to a gradual decrease in TBARS and H_2O_2 content in both *m. soleus* and *m. gastrocnemius* of rats (Figure 4a,b).

Thus, on day 5, the content of secondary LPO products and hydrogen peroxide in *m. gastrocnemius* decreased by 28% and 44%, respectively, and in *m. soleus* by 29% and 40%, respectively, compared with the first day of the experiment. The observed inhibition of excessive accumulation of LPO products and oxygen derivative H_2O_2 in both muscle types may be mediated by the antiradical properties of C_{60} fullerene. The results confirm that C_{60} fullerenes can penetrate through plasma membranes and accumulate in particular tissues, including muscles, without signs of damage [19, 41].

Antioxidant defense enzymes such as SOD, CAT, and GP_x play a key role in the mechanisms of regulation of free radicals and peroxide processes. SOD is the most powerful natural antioxidant and the enzyme of the first link of antioxidant protection, which carries out the dismutation reaction of superoxide anion radicals and converts them into less reactive hydrogen peroxide molecules [42].

Previous studies have shown [43] that SOD activity and protein accumulation in rat skeletal muscles increased during exercise without significant changes in mRNA expression. At the same time, SOD activity increased predominantly in oxidative muscles with a high content of type I and IIa fibers [44]. In our experiment (Figure 4c), during electrical stimulation of muscles against the background of C_{60} fullerene application, a gradual decrease in SOD activity was found in *m. gastrocnemius* (by 13%) on day 5, while in *m. soleus* SOD activity increased from day 3–4 (by 27% and 21%, respectively), and on day 5 only tended to increase relative to the first day of the experiment.

Physical exercise against the background of long-term administration of C_{60} fullerene did not lead to significant changes in CAT activity in both *m. gastrocnemius* and *m. soleus* during the 5 days of the experiment (Figure 5a). CAT activity significantly decreased in *m. gastrocnemius*, by 33% on day 5 of the experiment compared with day 1, while in *m. soleus* the activity of this enzyme only tended to decrease. Despite a considerable number of studies, the data on the activity of such endogenous antioxidants as CAT and GP_x during intensive physical activity of skeletal muscle remain contradictory. It was found that chronic exercise does not change CAT activity in slow muscles, but decreases it in fast muscles [45]. CAT activity has also been shown to decrease in both oxidative and glycolytic muscle fiber types [46]. In contrast, other studies have shown an increase in CAT activity [47].

Cellular mechanisms of antioxidant protection are also associated with the functioning of a powerful glutathione link [13]. Along with antiradical enzymes, the glutathione system is one of the active components of the body's antioxidant defense, which plays a significant role in the attenuation of the pathological process during muscle fatigue, since it not only prevents the free-radical reactions but also provides effective elimination of the final metabolites of LPO. The protective functions of GSH during oxidative stress are determined by the ability to catalyze the cleavage of hydrogen peroxide and fatty acid hydroperoxide with GSH [48].

In our study, after administration of C_{60} fullerenes against the background of fatigue development for 5 days, GP_x activity in *m. soleus* gradually decreased by 15%. At the same time, in *m. gastrocnemius* the activity of this anti-peroxide enzyme during the same period had a tendency only to decrease (Figure 5b). It is important to note that GP_x activity in *m. soleus* was three times higher than in *m. gastrocnemius*. This coincides with previous studies showing a significant increase in GP_x activity in oxidative muscle fibers under physical strain [45, 49]. At the same time, the mRNA level of GP_x expression in these muscles corresponded to the degree of activity of this enzyme [50].

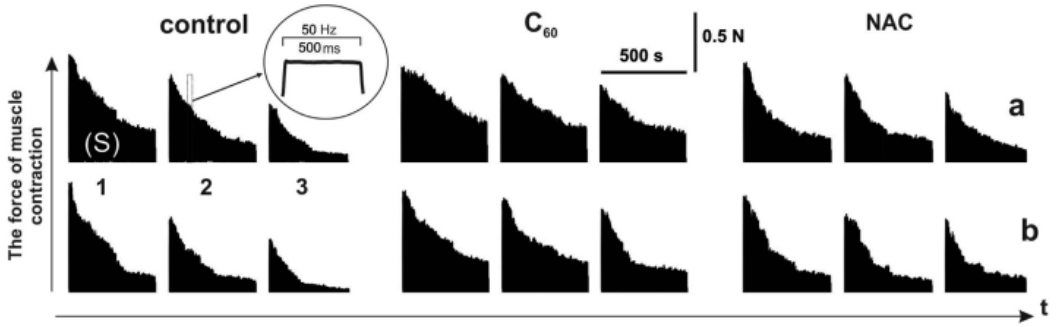

Figure 2. Contraction force of rat *m. soleus* (a) and m. gastrocnemius (b) after application of 50 Hz stimulation for 5 s with three successive pools: 1, 2, 3 - consecutive stimulation pools of 500 s duration each with 5 min relaxation between them; control - native muscle; C_{60} - muscle mechanograms 1 h after C_{60}FAS injection; NAC - muscle mechanograms 1 h after N-acetylcysteine injection; S - integrated muscle power (calculated area under the force curve).

The tendency established in our experiments to decrease the activity of antiradical (SOD) and anti-peroxide (CAT and GP_x) enzymes when using C_{60} fullerene in the development of muscle fatigue confirm the slowdown of oxidative processes in skeletal muscles of both types. Such dynamics of the activity of the above enzymes can testify to the efficiency of dismutation processes with a decrease in the level of aggressive superoxide radical, as well as the processes of elimination of peroxide compounds in rat muscle fibers [12].

Glutathione belongs to the main links of antioxidant protection, participating in the detoxification of xenobiotics and toxic metabolic products, the process of apoptosis, affects enzyme activity and nucleic acid biosynthesis, and regulates eicosanoids, and prostaglandins exchange [13, 48]. The use of C_{60} fullerene against the background of muscle fatigue caused the GSH content in *m. gastrocnemius* and *m. soleus* to increase by 51% and 61%, respectively, on the 5th day of the experiment compared to the 1st day (Figure 5c). This agrees with previous studies [51], which found that C_{60} fullerene can influence the processes of glutathione synthesis and metabolism in different tissues, including muscle, under different pathological conditions by modulating the Nrf2/ARE-antioxidant pathway. We also showed that C_{60}

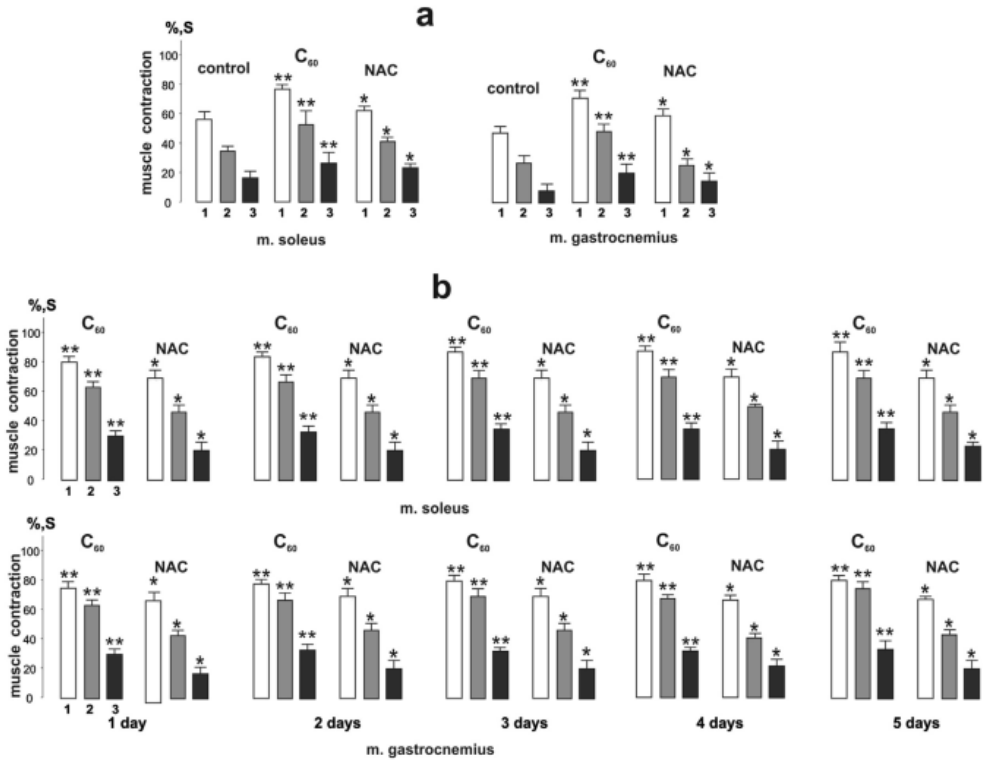

Figure 3. Integrated rat *m. soleus* and *m. gastrocnemius* power (*S*), presented as a percentage of maximum values after application of 50 Hz stimulation for 5 s in three successive pools (1, 2, 3) lasting 500 s each with 5 min of relaxation between them: muscle integrated power values 1 h after C_{60}FAS (C_{60}) and NAC administration (a); muscle integrated power values 2 h after C_{60}FAS (C_{60}) and NAC administration during 1st, 2nd, 3rd, 4th and 5th days (b). $^{*}p < 0.05$ compared to control (fatigue) value; $^{**}p < 0.05$ compared to value in NAC group; $n = 7$ in each group.

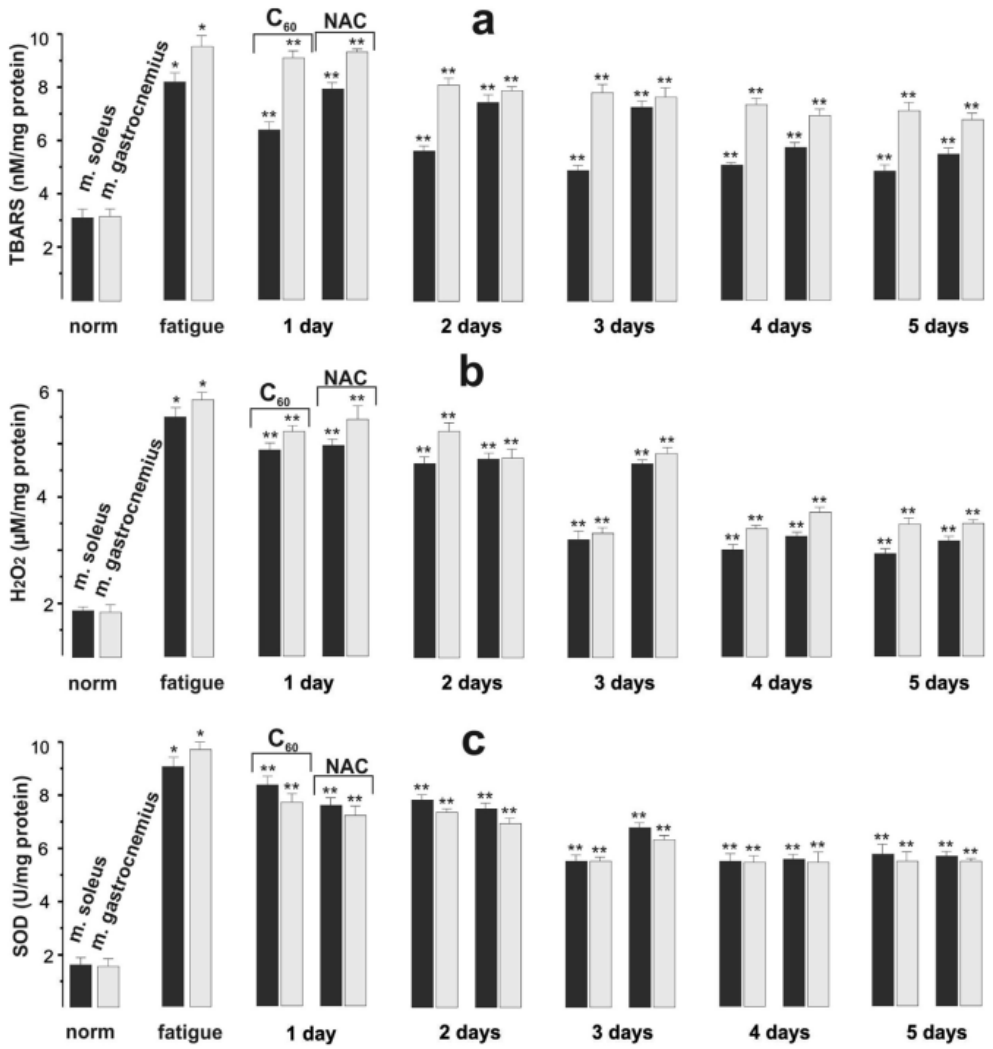

Figure 4. Indices of pro- and antioxidant balance (TBARS (a), H_2O_2 (b), and SOD (c)) in the studied rat muscle tissues after application of three-component stimulation and administration of C_{60}FAS (C_{60}) and NAC for 1,2,3,4 and 5 days. *$p < 0.05$ relative to the intact (norm) group; **$p < 0.05$ relative to the control (fatigue) group; $n = 7$ in each group.

fullerenes prevent mitochondrial dysfunction by restoring the activity of enzymes of mitochondrial complexes, as well as inhibiting mitochondrial-dependent apoptosis by limiting mitochondrial translocation of the p53 protein and increasing the expression of the Bcl-2 protein [52]. Consequently, the use of C_{60} fullerene led to an increase in the efficiency of the antioxidant defense system due to an increase in the GSH content in both slow and fast muscles, thereby increasing their resistance to physical activity.

If we compare the dynamics of changes in pro- and antioxidant homeostasis parameters between fast and slow muscles, it should be noted that the intensity of free-radical processes in these muscles is determined primarily by the peculiarities of their metabolism, functional load, and the level of antioxidant protection system [16]. It is known that during physical exercise the activity of SOD, GP_x, CAT enzymes, as well as GSH content, are the highest in oxidative muscles (type I and type IIa) that have an increased blood supply, much myoglobin, a large number of mitochondria, and energy supply mainly due to oxidative phosphorylation processes [53]. In our study, when C_{60} fullerene was applied during the development of muscle fatigue, the intensity of oxidative processes as well as the level of the antioxidant defense system in the fast and slow muscles of rats differed from each other. TBARS and H_2O_2 content in slow muscles were (70–71)% and (29–41)% higher than in fast muscles, respectively. Moreover, in slow muscles, the activity of anti-peroxide enzymes and GSH content exceeded those in fast muscles, and the extent of this increase was different. Thus, in slow muscles, CAT activity and GSH content were 8 and 6% higher, respectively, than in fast muscles. In slow muscles, GP_x activity was three times greater than in fast muscles. This tendency of the flow of antioxidant processes coincides with the data [50] regarding the difference in the activity and levels of mRNA expression of CAT and GP_x between fast and slow muscles at rest and after heavy exercise. However, our experiments showed that SOD activity in fast muscles was 35% higher than in slow muscles when C_{60} fullerene was applied.

Thus, the long-term use of C_{60} fullerene slows down the course of oxidative stress in fast and slow muscles by maintaining the balance between pro-oxidants and the antioxidant defense system, which

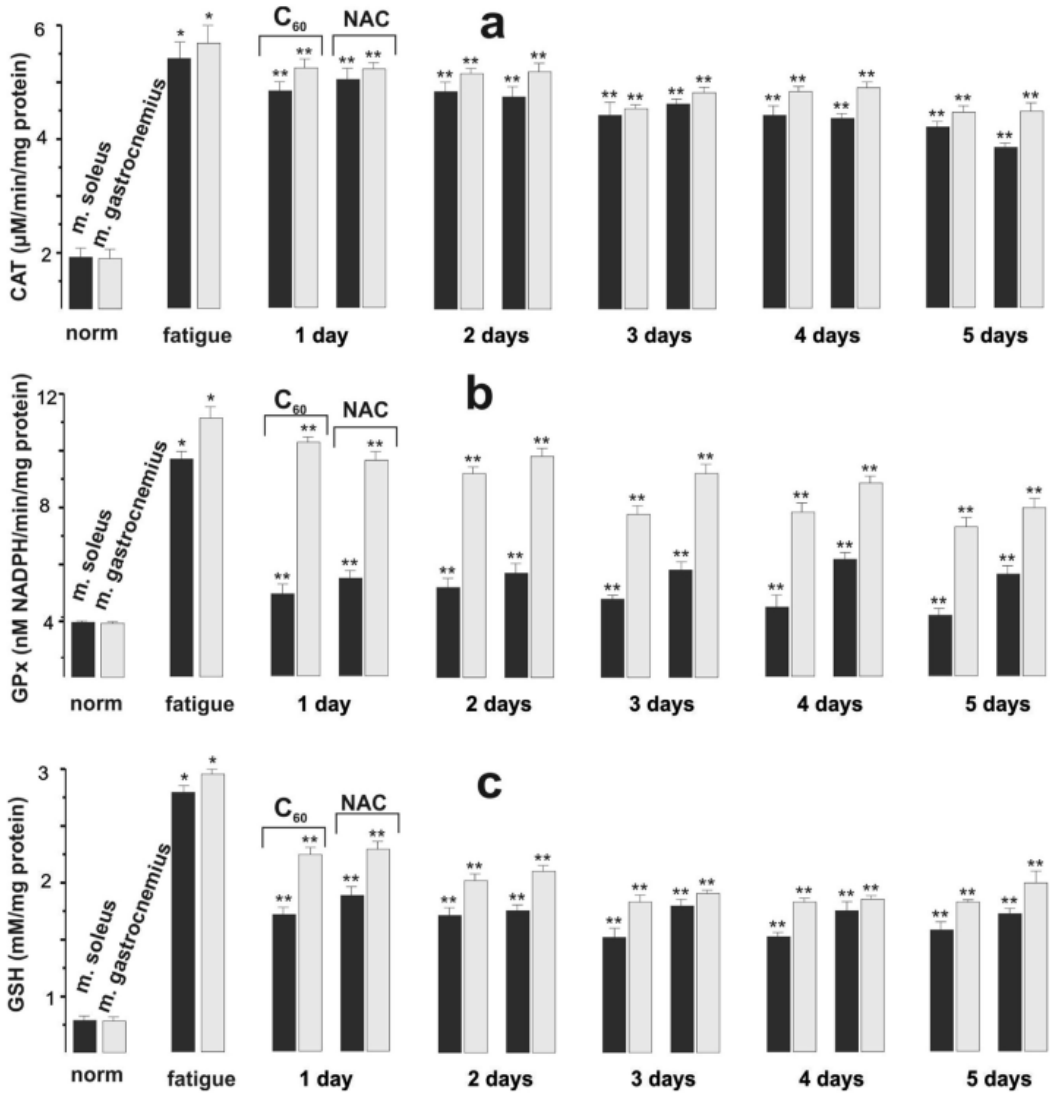

Figure 5. Indices of antioxidant defense (CAT (a), GPx (b), and GSH (c)) in the studied rat muscle tissues after the initiation of three-component stimulation and administration of C_{60}FAC (C_{60}) and NAC for 1,2,3,4 and 5 days. *$p < 0.05$ relative to the intact (norm) group; **$p < 0.05$ relative to the control (fatigue) group; $n = 7$ in each group.

prevents the negative effects of ROS on cellular and subcellular structures while muscle fatigue development in rats.

According to the literature data, NAC as a known glutathione precursor can neutralize free radicals and thus exhibit antiradical and antioxidant properties [54]. In addition, numerous studies have shown that the use of NAC removes muscle fatigue during submaximal exercise in humans, including electrical muscle stimulation [55]. Therefore, in our study, we used NAC as a comparison drug. NAC administration during electrostimulation of the rat muscles for 5 days also caused a decrease in TBARS and H_2O_2 content in fast muscles by 42% and 44%, respectively, and in slow muscles by 38% and 48%, respectively, compared with the first day of the experiment. The obtained data coincide with the results of other authors who showed the effectiveness of NAC in the elimination of ROS [56]. NAC application in the simulation of muscle fatigue caused a 29% decrease in CAT activity in fast muscles on day 5, while in slow muscles we registered only a decreasing tendency. SOD activity did not undergo significant changes in the two types of muscles studied throughout the experiment. At the same time, GSH content and GP_x activity in the fast muscles increased by 30% and 43%, respectively, and in the slow muscles by 21% and 34%, respectively, on day 5.

A comparative analysis of oxidative stress markers and indicators of the state of antioxidant defense systems showed that the protective effect of C_{60} fullerene was higher on the first day compared to NAC by (5–10)% in fast and slow muscles, increased to (20–35)% after 3 days of drug use and additionally increased by (8–9)% to 5th day.

4. Conclusions

The analysis of the obtained data showed positive dynamics of changes in the force of muscle contraction, markers of oxidative stress, and indicators of the state of antioxidant protection systems in fast and

slow muscles of rats with the intraabdominal injection of C_{60} fullerene and NAC at low doses of 1 and 150 mg kg^{-1}, respectively, as potential correctors of the effects of muscle fatigue. So, the effect of C_{60} fullerene on fast muscles was (15–17)% more effective than on slow muscles in terms of muscle force response against the background of fatigue development 1 h after C_{60} fullerene administration. Analysis of mechanograms 2 h after drug injection revealed a further slight increase in the effect of C_{60} fullerene, namely by (7–9)% in *m. gastrocnemius* and (5–6)% in *m. soleus*. The maximum effect was observed on the 1st day of the experiment. The increase C_{60} fullerene effect occurred within 4 days (the difference between the 4th and 5th day did not exceed (3–5)%) and exceeded the effect of NAC by (32–34)% overall.

The analysis of biochemical parameters in rat muscle tissues against the background of induced muscle fatigue showed that long-term application of C_{60} fullerene (for 5 days) slows down the course of oxidative stress by (10–30)% in fast muscles and by (5–20)% in slow muscles due to maintaining a balance between pro-oxidants and antioxidant defense system. The maximum decrease in biochemical markers was recorded after 3 daily administrations of drugs. At the same time, the protective effect of C_{60} fullerene was higher compared to NAC by (20–35)%, and this difference additionally increased by (8–9)% to the 5th day.

Thus, the above data indicate the prospect of using water-soluble C_{60} fullerenes, whose antioxidant effect exceeds the effect of the known compound NAC, as potential protective nanoagents to improve the efficiency of skeletal muscle function by modifying the ROS-dependent mechanisms that play an important role in the development of fatigue processes.

Declarations

Author contribution statement

Dmytro Nozdrenko, Kateryna Bogutska, Olga Gonchar, Svitlana Prylutska, Daria Franskevych, Yuriy Prylutskyy, Bohdan Hromovyk, Peter Scharff and Uwe Ritter: Conceived and designed the experiments, Performed the experiments, Analysed and interpreted the data, Contributed reagents, materials, analysis tools or data, Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no competing interests.

Additional information

No additional information is available for this paper.

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Research Article

The Residual Effect of C_{60} Fullerene on Biomechanical and Biochemical Markers of the Muscle Soleus Fatigue Development in Rats

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Muscle fatigue as a defense body mechanism against overload is a result of the products of incomplete oxygen oxidation such as reactive oxygen species. Hence, C_{60} fullerene as a powerful nanoantioxidant can be used to speed up the muscle recovery process after fatigue. Here, the residual effect of C_{60} fullerene on the biomechanical and biochemical markers of the development of muscle soleus fatigue in rats for 2 days after 5 days of its application was studied. The known antioxidant *N*-acetylcysteine (NAC) was used as a comparison drug. The atomic force microscopy to determine the size distribution of C_{60} fullerenes in an aqueous solution, the tensiometry of skeletal muscles, and the biochemical analysis of their tissues and rat blood were used in this study. It was found that after the cessation of NAC injections, the value of the integrated muscle power is already slightly different from the control (5%–7%) on the first day, and on the second day, it does not significantly differ from the control. At the same time, after the cessation of C_{60} fullerene injections, its residual effect was 45%–50% on the first day, and 17%–23% of the control on the second one. A significant difference (more than 25%) between the pro- and antioxidant balance in the studied muscles and blood of rats after the application of C_{60} fullerene and NAC plays a key role in the long-term residual effect of C_{60} fullerene. This indicates prolonged kinetics of C_{60} fullerenes elimination from the body, which contributes to their long-term (at least 2 days) compensatory activation of the endogenous antioxidant system in response to muscle stimulation, which should be considered when developing new therapeutic agents based on these nanoparticles.

1. Introduction

Muscle fatigue is usually a short-term and reversible process manifested as a lack of energy [1]. The main causes of fatigue are related to overexertion, insufficient relaxation time, or physical trauma [2, 3]. However, muscle fatigue can be persistent and more severe when associated with pathological conditions, or chronic exposure to certain drugs and toxic compounds [4–6]. Although the origin of fatigue is multifactorial [7], muscle fatigue in pathological states is inextricably linked to the occurrence of muscle-mass loss and difficulty in performing precise goal-directed movements [8].

Fatigue can be caused by many mechanisms, ranging from metabolite accumulation in muscle fibers [9] to inadequate motor command generation [10]. Therefore, there is no single mechanism responsible for muscle fatigue; the mechanisms that cause fatigue are specific to the particular

task performed [11]. Quantitatively, the development of muscle fatigue is defined as a decrease in maximum muscle strength or power, meaning that maximum contractions cannot be maintained after the onset of muscle fatigue [12].

Any stress response of an organism is accompanied by an increase in reactive oxygen species (ROS). Today it is believed that free-radical processes may occupy one of the key positions in the regulatory mechanisms that determine the possibility of cell survival, its death, or transformation in stressful situations [13]. The condition in which the generation of free-radical processes increases more than the capacity of the antioxidant system due to the action of any factors is defined by researchers as oxidative stress [14]. Literature evidence suggests that free radicals are the main pathogenic factor in the process of muscle fatigue [15, 16]. They include the initiation of lipid peroxidation (LPO), direct inhibition of mitochondrial respiratory chain enzymes and ATPase (adenosine 5′-triphosphatase) activity, inactivation of glyceraldehyde-3-phosphate dehydrogenase, and membrane sodium channels [17, 18]. When stress is of sufficient strength, oxidative stress acts as a serious danger to the functioning of the organism, so there is a complex multilevel protection system against the excessive formation of free-radical transformation products in all cellular components [19]. The link of antioxidant reactions in the mechanism of protective processes is leading and most powerful, because it not only prevents the development of free-radical reactions, and the accumulation of superoxide anions, and peroxide, but also maintains the high activity of redox processes, provides elimination of final oxygen metabolites, promotes the activity of synthetic processes, including antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP_x), which form, respectively, the first and second lines of protection [20].

At the moment, the search and development of new drugs are being actively conducted to reduce the manifestation of fatigue processes in skeletal muscles, changes in the biochemical parameters of the oxidative status, and energy metabolism under their influence. The main biochemical markers of the therapeutic effect of the drugs are the content of lactic acid, creatine, creatine kinase, SOD, and GP_x [20, 21]. However, the concentration of these markers in the blood and fatigued muscles can differ significantly and, thus, the interpretation of the kinetics of the therapeutic effect of the studied drug is much more difficult [21]. Biocompatible carbon nanostructures, C_{60} fullerenes, can be considered potential antioxidants [22]. They easily attach up to six electrons and thus can act as effective free radical scavengers [23]. We previously tested the powerful antioxidant properties of C_{60} fullerenes in experiments on ischemia, fatigue, and skeletal muscle injury [24–26]. However, the development of fatigue involves processes at all levels of the motor pathway between the brain and muscles [3]. Central fatigue is the inability of the nervous system to maximize muscle control [7]. It is defined as a progressive decrease in arbitrary activation or nerve impulse to a muscle caused by exercises [8]. On this basis, it cannot be excluded that the components of the applied drug on the nerve conduction of afferents may contribute to the resulting therapeutic effects [4, 5, 10, 25, 26] and thus contribute to improving the kinetics of neuronal mediator recovery. Since the question of the duration of the therapeutic effect after the agent application remains important within the framework of an adequate analysis of the dynamics of the muscle antifatigue therapy, we studied the residual effect of C_{60} fullerene on biomechanical and biochemical markers of fatigue development in rat muscle soleus during 2 days after its 5-day application. The known antioxidant *N*-acetylcysteine (NAC) [27, 28] was used as a comparison drug.

2. Materials and Methods

2.1. Preparation and Characterization of C_{60}FAS. A highly stable reproducible C_{60} fullerene aqueous colloid solution (C_{60}FAS) was prepared according to the protocol [29, 30]. Briefly, for the preparation of C_{60}FAS, we used a saturated solution of pure C_{60} fullerene (purity >99.99%) in toluene with a C_{60} molecule concentration corresponding to maximum solubility near 2.9 mg mL^{-1}, and the same amount of distilled water in an open beaker. The two phases formed were treated in an ultrasonic bath. The procedure was continued until the toluene had completely evaporated and the water phase became yellow-colored. Filtration of the aqueous solution allowed to separate the product from undissolved C_{60} fullerenes. The concentration of C_{60} fullerene in the prepared C_{60}FAS sample was determined as the concentration of total organic carbon in an aqueous solution (Analytik Jena TOC Analyser multi N/C 3100). In our experiments, the C_{60}FAS sample with 0.15 mg mL^{-1} concentration of C_{60} fullerene was used. The prepared C_{60}FAS is stable within 12–18 months at temperature +4°C.

The atomic force microscopy (AFM) was performed to determine the size of C_{60} fullerene particles (their aggregates) in an aqueous solution [31]. Measurements were done with the "Solver Pro M" system (NT-MDT, Russia). A drop of investigated solution was transferred to the atomic-smooth substrate to deposit layers. Measurements were carried out after complete evaporation of the solvent. For AFM studies, a freshly broken surface of mica (SPI supplies, V-1 grade) was used as a substrate. Measurements were carried out in a semicontact (tapping) mode with AFM probes of the RTPE-SPA150 (Bruker, 6 N/m, 150 kHz) type.

2.2. Animals. Male Wistar rats (170 ± 12 g, 2 months old) were bred and housed in standard temperature conditions (21–23°C), lighting (12/12 hr light–dark cycle), and humidity (30%–35%). All animals had unlimited access to chow and tap water. The study was carried out in strict accordance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986) and was approved by the Bioethical Committee of the ESC "Institute of Biology and Medicine" of the Taras Shevchenko National University of Kyiv, Ukraine (study protocol no. 10 dated October 20, 2021).

The following groups of animals were tested: two experimental groups after 5 days of C_{60}FAS ($n = 7$) and NAC ($n = 7$) administration on the 1st and 2nd day after the cessation of the respective drug injection; the control ("fatigue,"

saline administration) group ($n = 7$) and the intact ("norm," no fatigue) group ($n = 7$).

Based on previously obtained data [28], the research protocol involved intraperitoneal injection of C_{60}FAS and NAC at a daily dose of 1 and 150 mg kg^{-1}, respectively, 1 hr before the experiment for 5 days.

It is important to note that the selected dose of C_{60}FAS in our experiments is significantly lower than the LD_{50} (lethal dose) value, which was 600 mg kg^{-1} body weight when administered orally to rats [32] and 721 mg kg^{-1} when administered intraperitoneally to mice [33].

2.3. Biomechanical Analysis. The object of the study was the rat muscle soleus. In preliminary preparation for the experiment, anesthesia was performed by intra-abdominal injection of Nembutal (40 mg kg^{-1}). Standard preparation included cannulation (a. carotis communis sinistra) for pressure measurement and laminectomy at the lumbar spinal cord level. Muscle soleus was released from surrounding tissues, and their tendon parts were connected to force measurement sensors in the distal part. To prepare for modulated efferent stimulation, the ventral roots in the respective segments were transected directly at their exit points from the spinal cord.

The dynamic properties of muscle contraction were studied under conditions of muscle activation using the method of modulated efferent stimulation [34]. Fatigue was induced by successive stimulation impulses with a frequency of 50 Hz and a duration of 5 s each, without a relaxation period between them. The sum of such stimulation signals was 500 s, followed by 5 min of relaxation. The number of stimulation pools was three. The external load on the muscle was controlled by using a system of mechanostimulators. Changes in contraction force were measured by strain gauges. During the analysis of the results, we used quantitative parameters-integrated muscle power (calculated area under the strength curve), which is an indicator of its general performance under the applied stimulation pools, and levels of maximum and minimum strength generation of contraction, which are indicators of the general dysfunction of the muscular system in the development of fatigue [4, 5, 24, 26].

2.4. Biochemical Analysis. LPO was measured from the formation of thiobarbituric acid-reactive substances (TBARS) using the method of Buege and Aust [35]. TBARS were isolated by boiling tissue homogenates for 15 min at 100°C with thiobarbituric acid reagent (0.5% 2-thiobarbituric acid/10% trichloroacetic acid/0.63 mM (millimolar) hydrochloric acid) and measuring the absorbance at 532 nm. The results were expressed as nM (nanomolar) mg^{-1} of protein using $\varepsilon = 1.56 \times 10^5$ mM^{-1} cm^{-1}.

The hydrogen peroxide (H_2O_2) concentration in the tissue homogenates was measured using the method, which is based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange (*o*-cresolsulphonephthalein 3′,3″-bis(methylimino) diacetic acid, sodium salt). This method is extremely sensitive and is used to measure low levels of water-soluble hydroperoxide present in the aqueous phase. To determine the H_2O_2 concentration, 500 μL of the incubation medium was added to 500 μL of assay reagent (500 μM ammonium ferrous sulfate, 50 mM H_2SO_4, 200 μM xylenol orange, and 200 mM sorbitol). The absorbance of the Fe^{3+}-xylenol orange complex (A560) was detected after 45 min. Standard curves of H_2O_2 were obtained for each independent experiment by adding variable amounts of H_2O_2 to 500 μL of basal medium mixed with 500 μL of an assay reagent. Data were normalized and expressed as μM H_2O_2 per mg protein [36].

Total SOD activity was measured by the method [37], which is based on the inhibition of autooxidation of adrenaline to adrenochrome by SOD contained in the examined samples. The results were expressed as specific activity of the enzyme in units per mg protein. One unit of SOD activity is defined as the amount of protein, causing 50% inhibition of the conversion rate of adrenaline to adrenochrome, under specified conditions.

CAT activity was measured by the decomposition of hydrogen peroxide, determined by a decrease in the absorbance at 240 nm [38].

The activity of selenium-dependent GP_x was determined according to the method [39]. Briefly, the reaction mixtures consisted of 50 mM KPO_4 (pH 7.0) 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM NaN_3, 0.2 mM NADPH (nicotinamide adenine dinucleotide phosphate), 1 mM GSH (reduced glutathione), 0.25 mM H_2O_2, 226 U mL^{-1} glutathione reductase, and rates of NADPH oxidation followed at 340 nm.

The GSH was determined as described [40]. The tissue sample was mixed with sulphosalicylic acid (4%) and incubated at 4°C for 30 min. Thereafter, it was centrifuged at 1,200 × g for 15 min at 4°C and 0.1 mL of this supernatant was added to phosphate buffer (0.1 M, pH 7.4) containing DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid) in abs. ethanol. The yellow color developed was read immediately at 412 nm. The GSH content was calculated as mM GSH/mg protein ($\varepsilon_{412} = 13.6 \times 10^3$ M^{-1} cm^{-1}).

The levels of TBARS, hydrogen peroxide, GSH, GP_x, SOD, and CAT activity as markers of total antioxidant status [41], were determined in the blood plasma of experimental animals using clinical diagnostic equipment—a haemoanalyzer (Erba, Czech Republic) [24].

2.5. Statistical Analysis. Statistical processing of the measurement results was performed by methods of variational statistics using the software Origin 9.4. Each of the experimental force curves obtained in the work is the result of averaging 10 similar experiments. At least three repetitions were performed for each biochemical measurement. Data are expressed as means ± SEM (standard error of the mean) for each group. Differences from experimental groups were indicated by one-way ANOVA described Bonferroni's multiple comparison test. Values of $p < 0.05$ were considered significant.

3. Results and Discussion

3.1. Characterization of C_{60}FAS. When studying the layers deposited from C_{60}FAS, 3D images of AFM revealed a monodisperse film (~1.1 nm thick) in the form of a solid mesh

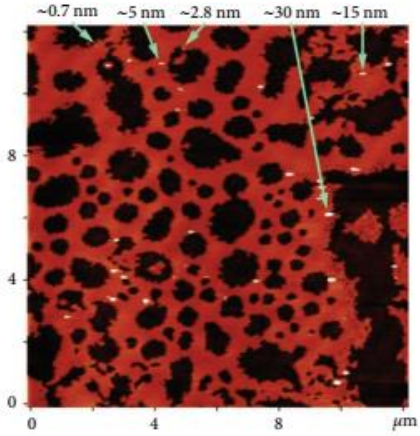

FIGURE 1: AFM image of the C_{60} fullerene layer deposited from C_{60}FAS (0.15 mg mL^{-1}) on the mica surface. Arrows indicate the heights of nanoscale objects.

superstructure and individual islands (Figure 1). As one can see, in addition to individual C_{60} molecules (a height of ~0.7 nm), its composition includes nanoaggregates of C_{60} fullerenes with a height of 2.8–30 nm. It is important to note that the size distribution of C_{60} fullerene nanoparticles in an aqueous solution did not change for 6 months, which indicates the absence of additional aggregation of nanoparticles and confirms their suitability for in vivo studies.

3.2. Biomechanical Analysis. The phenomenological approach to the analysis of pathological processes affecting the mechanical properties of the muscle makes it possible to establish important relationships between the macroscopic parameters of the muscle state, such as strength, speed, elasticity, and the level of afferent activity. In many cases, this is sufficient to analyze the processes of regulation of motor activity and the degree of the pathological condition and assess the impact of therapeutic measures.

Figure 2 shows the change in contractile strength of rat muscle soleus when applying 50 Hz stimulation for 5 s in three successive pools for 500 s each with 5 min of relaxation between them after C_{60}FAS and NAC administration for 5 days. The three-component muscle stimulation was chosen based on our earlier data [4, 5, 12, 25, 26, 28] on the maximum level of fatigue processes development exactly at this type of fatigue stimulation, which exhausts the possibilities of adaptive restructuring of the muscle system to great physical loads and implies the presence of the full range of muscle fatigue dysfunctions.

Analysis of the value of integrated power allows us to evaluate the kinetics of muscle fatigue formation in the system of equilibrium "force of contraction-external load," which is a physiological analog of the performance of the muscular system as a whole (Figure 3(a)). It turned out that already after the first pool of stimulation, integrated power decreased significantly by $58 \pm 4\%$. After the relaxation period, it decreased progressively at the 2nd and 3rd stimulation pools and was $39 \pm 2\%$ and $24 \pm 3\%$ of control values, respectively.

Application of NAC during 5 days increased the value of this index: its effect was $32 \pm 3\%$, $28 \pm 2\%$, and $25 \pm 2\%$ at the 1st, 2nd, and 3rd stimulation pools, respectively. On the second day after cessation of NAC therapy, its residual effect was no more than 5% of control values at the 1st, 2nd, and 3rd stimulation pools and remained virtually unchanged thereafter.

Injection of C_{60}FAS increased the level of this parameter. On day 5 of therapy, its effect was $41 \pm 3\%$, $69 \pm 6\%$, and $75 \pm 3\%$ at the 1st, 2nd, and 3rd stimulation pools, respectively. On the second day after cessation of C_{60}FAS injection, its residual effect was 39%, 30%, and 25% of the control values at the 1st, 2nd, and 3rd stimulation pools, respectively, and did not change significantly thereafter.

Changes in the levels of generation of maximum and minimum contraction strength can be associated both with the development of fatigue processes in the neural component and with myotic components of the pathology under study. When performing sufficiently simple single-joint movements, these markers are the main indicators of muscle dysfunction, the phenomenological analysis of which makes it possible to establish the presence of causal relationships between the level of decrease in the biomechanical activity of the muscles, the main mechanical parameters of movements, and the level of development of the pathological process.

The analysis of the obtained mechanograms (Figure 3(b)) showed changes in the maximum strength of contractions in the control measurements, which were 0.7 ± 0.1 N, 0.42 ± 0.03 N, and 0.39 ± 0.03 N at 1st, 2nd, and 3rd stimulation pools, respectively.

The use of NAC did not significantly change the maximum contraction strength at any of the three studied stimulation pools during the 5 days of its application. On the first day after discontinuation of NAC, its residual effect was 8%, 6%, and 5% on 1st, 2nd, and 3rd pools, respectively, and on the second day, no significant differences in mechanokinetics of muscle contraction were recorded

The use of C_{60}FAS for 5 days increased the described values. Its residual effect was 39%, 26%, and 21% at 1st, 2nd, and 3rd pools, respectively, on day 1 after discontinuation of the agent and 16%, 9%, and 6% on day 2.

The minimum force response of the studied muscle in the control (Figure 3(b)) was 0.50 ± 0.04 N, 0.21 ± 0.02 N, and 0.10 ± 0.01 N at the 1st, 2nd, and 3rd pools, respectively.

Application of NAC injections during 5 days increased minimum strength values. On the first day after cessation of NAC use, its residual effect was 8% and 5% at 1st and 2nd pools, respectively, and at 3rd pool, as well as on day 2 after cessation of NAC use, no significant differences in mechanokinetics of contraction were recorded.

Injections of C_{60}FAS during 5 days have significantly changed the mechanokinetics of the contractile process: its effect was 44%, 80%, and 470% at the 1st, 2nd and 3rd pools, respectively. As can be seen, the maximum effect is observed on the 3rd pool of contraction, on which, in turn, the most severe disturbances of contraction biomechanics are observed

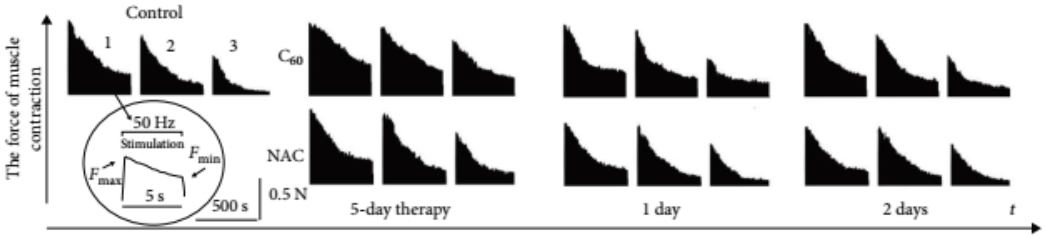

FIGURE 2: Recording the contractile force of rat muscle soleus when applying 50 Hz stimulation for 5 s in three consecutive pools (1, 2, and 3) for 500 s each with 5 min relaxation between them: control-native muscle; 5-day therapy—mechanograms of muscle after C_{60}FAS (C_{60}) and NAC administration during 5 days; 1 day and 2 days—mechanograms of muscle on day 1 and 2, respectively, after C_{60} and NAC administration; F_{max} and F_{min}—maximum and minimum strength of a single contraction, respectively.

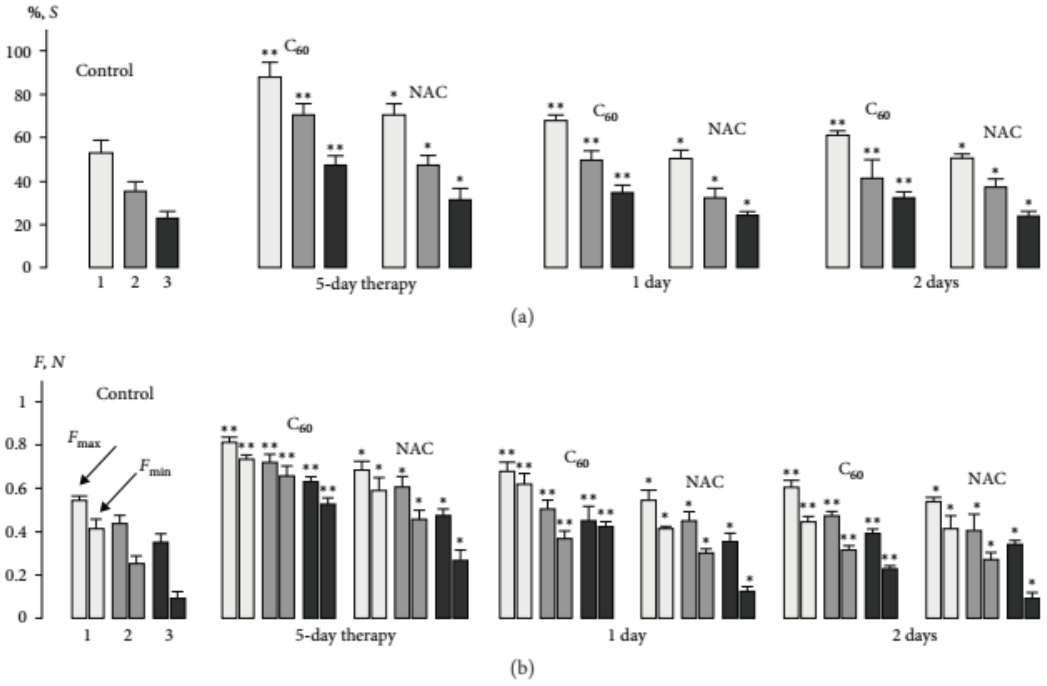

FIGURE 3: Integrated power (S, presented as a percentage of maximum values) (a) and peak contraction force values (F, N) (b) of rat muscle soleus when applied 50 Hz stimulation for 5 s duration in three consecutive pools (1, 2, and 3) for 500 s duration each with 5 min relaxation between them: control-native muscle; 5-day therapy—muscle mechanograms after C_{60}FAS (C_{60}) and NAC administration for 5 days; 1 day and 2 days—muscle mechanograms on day 1 and 2, respectively, after C_{60} and NAC administration; F_{max} and F_{min}—maximum and minimum single contraction force, respectively. $^{*}p<0.05$ compared with control ("fatigue" group); $^{**}p<0.05$ compared to values in the NAC group.

in the development of skeletal muscle fatigue. The residual effect of C_{60}FAS was 37%, 42%, and 227% on pools 1, 2, and 3, respectively, on the first day after discontinuation of the drug and 15%, 19%, and 158% on day 2.

Thus, the biomechanical effects of skeletal muscle described above are based on the antioxidant mechanism of C_{60} fullerene action. The increase in muscle contraction strength is probably due to the inactivation of the excess amount of secondary oxidation products in muscle fibers by C_{60} fullerenes, which leads to a slowdown in the development of the fatigue process. The use of C_{60} fullerene therapy can reduce the severity of muscle fiber microtraumas that occur during prolonged physical exertion. As is known, the main factor in membrane damage is an increase in LPO induced by an excess of free radicals. The appearance of a large number of injured myocytes leads to the occurrence of local inflammatory processes and, as a result, an increase in subfascial pressure and the occurrence of myofascial compartment syndrome. These processes increase the stiffness components of the muscles, leading to a decrease in the time of fatigue development and a significant decrease in

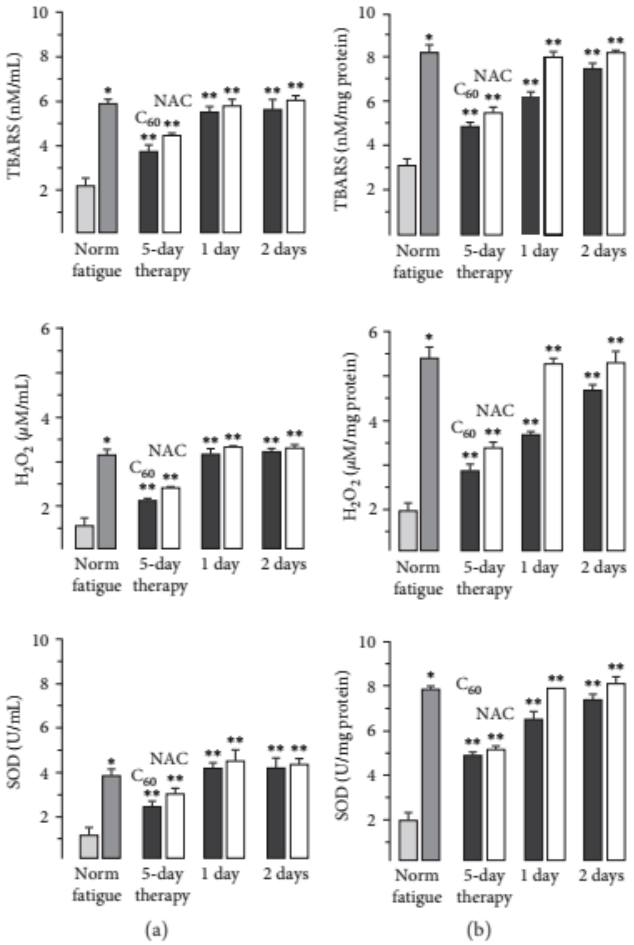

FIGURE 4: Indicators of pro- and antioxidant balance (TBARS, H_2O_2, and SOD) in the blood plasma (a) and muscle soleus (b) of rats after C_{60}FAS and NAC application: 5-day therapy—data after C_{60}FAS (C_{60}) and NAC administration for 5 days; 1 day and 2 days—data on the first and second day, respectively, after C_{60} and NAC application. $^{*}p < 0.05$ compared with intact ("norm") group; $^{**}p < 0.05$ compared with control ("fatigue" group).

muscle power. To confirm this hypothesis, it is necessary to analyze changes in the parameters of the pro- and antioxidant balance of muscle tissue and blood in rats in the described models of the development of fatigue processes.

3.3. Biochemical Analysis. The changes in blood chemistry during the development of fatigue processes are a reflection of biochemical shifts occurring both in the muscle complex and at the level of accessory organs and tissues (activity of liver enzymes, kidney function, etc.). We compared antioxidant enzymes isolated from the blood of the studied animals and the tissues of the muscle subjected to stimulation fatigue.

Inflammatory processes occurring immediately after the onset of fatigue in skeletal muscle are a source of ROS and contribute to the intensification of LPO processes [34, 35]. This interferes with the adequate performance of muscle work and significantly increases the recovery period. During reperfusion, oxygen entering the tissues initiates xanthine and hypoxanthine oxidation by xanthine oxidase, which leads to the formation of large amounts of superoxide anion radicals and hydrogen peroxide. As a result of biochemical tests, we have determined the number of secondary LPO products and antioxidant levels in the blood of rats after induction of fatigue. The obtained data demonstrate an increased level of markers of peroxidation and oxidative stress after the occurrence of muscle fatigue and their decrease with the applied therapeutic agents (Figures 4 and 5).

The change in TBARS level in the blood plasma on day 5 of the experiment was $5.8 \pm 0.2\,\text{nM}\,\text{mL}^{-1}$ during the development of fatigue ($2.2 \pm 0.4\,\text{nM}\,\text{mL}^{-1}$ in the intact group), 4.4 ± 0.1 and $4.1 \pm 0.4\,\text{nM}\,\text{mL}^{-1}$ after application of NAC

and C_{60}FAS, respectively. On the second day after 5 days of NAC administration, its level was 5.2 ± 0.2 nM mL^{-1} (10% residual effect). After discontinuation of C_{60}FAS, its residual effect on the first and second days was 19% and 14%, respectively (Figure 4(a)).

The change of TBARS level in rat muscle soleus on day 5 of the experiment was 8.2 ± 0.1 nM mg^{-1} protein during fatigue development (2.5 ± 0.3 nM mg^{-1} protein in the intact group), 5.8 ± 0.1 nM mg^{-1} protein and 4.8 ± 0.2 nM mg^{-1} protein after NAC and C_{60}FAS application, respectively. On day 2 after a 5-day NAC administration, its level did not differ from the control values. After discontinuation of C_{60}FAS, its residual effect on the first and second days was 6.1 ± 0.2 nM mg^{-1} protein and 7.3 ± 0.3 nM mg^{-1} protein (26% and 11% effect), respectively (Figure 4(b)).

Blood plasma H_2O_2 levels on day 5 of the experiment were 3.3 ± 0.2 μM mL^{-1} during fatigue development (0.8 ± 0.1 μM mL^{-1} in the intact group), 2.4 ± 0.2 and 2.1 ± 0.2 μM mL^{-1} after NAC and C_{60}FAS administration, respectively. On the second day after 5 days of NAC administration, its level did not differ from the control values. After discontinuation of C_{60}FAS, its residual effect on the first and second days was 2.5 ± 0.1 and 2.8 ± 0.2 μM mL^{-1} (18% and 10% effect), respectively (Figure 4(a)).

The change of H_2O_2 level in rat muscle soleus on day 5 of the experiment was 3.3 ± 0.2 μM mg^{-1} protein during the development of fatigue (0.8 ± 0.1 μM mg^{-1} protein in the intact group), 4.4 ± 0.2 μM mg^{-1} protein and 2.7 ± 0.1 μM mg^{-1} protein after NAC and C_{60}FAS application, respectively. On the first day after 5-day NAC administration, its residual effect was 5%, and on the second day, the H_2O_2 level did not differ from the control values. After discontinuation of C_{60}FAS, its residual effect on was 3.6 ± 0.1 μM mg^{-1} protein and 4.7 ± 0.2 μM mg^{-1} protein (34% and 14% effect), respectively (Figure 4(b)).

Blood plasma SOD activity on day 5 of the experiment was 3.9 ± 0.1 U mL^{-1} during fatigue development (1.3 ± 0.1 U mL^{-1} in the intact group), 3.0 ± 0.2 and 2.3 ± 0.2 U mL^{-1} after NAC and C_{60}FAS administration, respectively. On the first day after a 5-day administration of NAC and C_{60}FAS, a 9% residual effect was observed only for C_{60}FAS. On the second day, the blood SOD activity was no different from the control values for both agents (Figure 4(a)).

The SOD activity in the muscle soleus on day 5 of the experiment was 6.8 ± 0.2 U mg^{-1} protein during fatigue development (1.6 ± 0.1 U mg^{-1} protein in the intact group), 5.0 ± 0.2 U mg^{-1} protein, and 4.1 ± 0.2 U mg^{-1} protein after NAC and C_{60}FAS administration, respectively. On the first and second days after discontinuation of NAC, SOD activities were virtually indistinguishable from control values, and C_{60}FAS showed a residual effect of 5.9 ± 0.1 U mg^{-1} protein and 6.2 ± 0.1 U mg^{-1} protein (13% and 8% therapeutic effect), respectively (Figure 4(b)).

The CAT activity in the blood plasma on day 5 of the experiment increased from 0.9 ± 0.1 μM min^{-1} mL^{-1} in the intact group to 4.3 ± 0.1 μM min^{-1} mL^{-1} after the development of muscle fatigue, decreasing to 3.4 ± 0.1 and 2.8 ± 0.1 M min^{-1} mL^{-1} with NAC and C_{60}FAS injections, respectively. On the first day after a 5-day application of NAC and C_{60}FAS, a residual effect of 7% was observed only for C_{60}FAS. On day 2, the activity of CAT in the blood no longer differed from the control values for both drugs (Figure 5(a)).

The CAT activity in the muscle soleus on day 5 of the experiment was 1.8 ± 0.1 μM min^{-1} mg^{-1} protein in the intact group, 4.6 ± 0.2 μM min^{-1} mg^{-1} protein after fatigue induction, 4.1 ± 0.1 μM min^{-1} mg^{-1} protein and 3.4 ± 0.3 μM min^{-1} mg^{-1} protein when applying NAC and C_{60}FAS, respectively. A residual effect (13%) was recorded only for C_{60}FAS on the second day after discontinuation of its use (Figure 5(b)).

The concentration of GSH in the blood plasma on day 5 of the experiment was 2.7 ± 0.6 mM mL^{-1} during the development of fatigue (1.2 ± 0.1 mM mL^{-1} in the intact group), 2.1 ± 0.6 and 1.6 ± 0.5 mM mL^{-1} after NAC and C_{60}FAS administration, respectively. The residual effect of NAC was 9% on the first day, and for C_{60}FAS it was 17% and 11% on the first and second day, respectively, after discontinuation of its administration (Figure 5(a)).

The GSH concentration in the muscle soleus on day 5 of the experiment was 1.2 ± 0.1 mM mg^{-1} protein in the intact group and increased to 2.6 ± 0.2 mM mg^{-1} protein during the development of fatigue, decreasing to 1.7 ± 0.1 mM mg^{-1} protein and 1.5 ± 0.1 mM mg^{-1} protein after NAC and C_{60}FAS, respectively. The residual effect of NAC was 8% on the second day, and for C_{60}FAS it was 27% and 17% on the first and second days, respectively, after discontinuation of its use (Figure 5(b)).

Cellular mechanisms of antioxidant protection are also associated with the functioning of a powerful glutathione link. The protective functions of GP_x during oxidative stress are determined by the ability to catalyze the cleavage of hydrogen peroxide and fatty acid hydroperoxide with GSH. The GP_x concentration in blood plasma on day 5 of the experiment was 3.9 ± 0.4 nM NADPH min^{-1} mL^{-1} in the intact group, 7.9 nM NADPH min^{-1} mL^{-1} after fatigue initiation, 6.4 ± 0.2 nM NADPH min^{-1} mL^{-1} and 5.9 ± 0.4 nM NADPH min^{-1} mL^{-1} after NAC and C_{60}FAS, respectively. The residual effect of NAC was 8% and 4% on the first and second days, respectively, and for C_{60}FAS it was 16% and 12%, respectively, after discontinuation of its administration (Figure 5(a)).

GP_x concentration in muscle soleus on day 5 of the experiment was 4.1 ± 0.4 nM NADPH min^{-1} mg^{-1} protein in the intact group, 8.9 ± 0.6 nM NADPH min^{-1} mg^{-1} protein after fatigue initiation, 5.3 ± 0.5 nM NADPH min^{-1} mg^{-1} protein and 5.9 ± 0.4 nM NADPH min^{-1} mg^{-1} protein after NAC and C_{60}FAS administration, respectively. The residual effect of NAC was 9% and 6% on the first and second days, respectively, and for C_{60}FAS was 26% and 16%, respectively, after discontinuation of its administration (Figure 5(b)).

Our studies have shown that on day 6 and 7 of the experiment (day 1 and 2 after cessation of the administration of the corresponding drug), SOD activity remains significant in the muscles of rats against the background of decreased LPO processes. This indicates the preservation of a sufficient level of aggressive superoxide anion dismutation in the remote period of the experiment. The decrease in H_2O_2 content under these conditions is evidence of the coordinated action of the antiperoxide enzymes, CAT and GP_x, whose

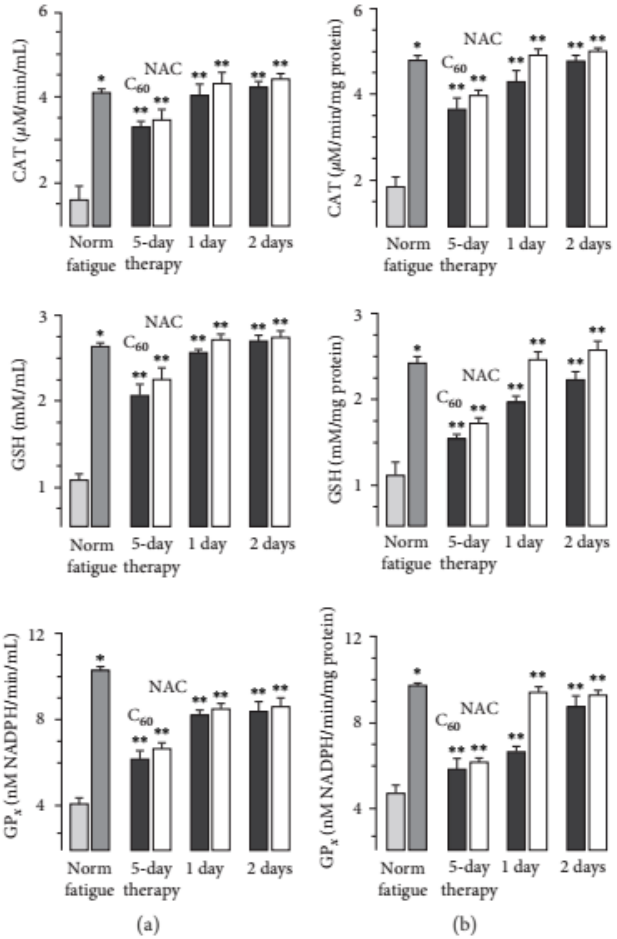

FIGURE 5: Indicators of antioxidant balance (CAT, GSH, and GP_x) in the blood plasma (a) and muscle soleus (b) of rats after C_{60}FAS and NAC application: 5-day therapy—data after C_{60}FAS (C_{60}) and NAC administration for 5 days; 1 day and 2 days—data on the first and second day, respectively, after C_{60} and NAC administration. $^{*}p < 0.05$ compared with intact ("norm") group; $^{**}p < 0.05$ compared with control ("fatigue" group).

activity also increases. This is probably due to the formation of adaptation reactions of the organism to the action of an extreme stimulus. Repeated induction of ROS during muscle stimulation results in increased cellular resistance to a stress factor and forms long-term adaptation [42]. Under these conditions ROS play the role of secondary messengers, participating in the processes of natural signal transduction in tissues. This is manifested primarily by the activation of transcription factors (hypoxia-inducible factor (HIF-1), nuclear factor kappa B (NF-κB), activator protein (AP-1)) and corresponding genes encoding antioxidant enzymes, in particular SOD, enzymes of the glutathione system, and CAT [42, 43]. Against this background, the use of C_{60} fullerenes can enhance and promote further activation of the above processes. This is proved by our preliminary studies, which confirmed the fact that the use of C_{60} fullerene under extreme conditions affects the rapid formation of adaptive reactions of the body by affecting such transcription factors as Nrf2 (NF-E2-related factor 2), NF-κB, and p53 [44]. The glutathione system plays an important role in the implementation of antiradical and antiperoxide cell protection [22]. The coordinated action of all components (GSH, glutathione-dependent, and NADPH-generating enzymes) contributes to the establishment of optimal levels of peroxide compounds and the preservation of antioxidant homeostasis [45]. Glutathione's high-redox activity with simultaneous resistance to oxygen oxidation, significant concentration in the cell, and ability to maintain its reduced state make it an important intracellular redox buffer [46]. Recent studies suggest a nonspecific nature of changes in the content of thiol compounds (primarily glutathione) for the action of extreme factors on the body, as well as their participation in the formation of adaptation processes [47]. Herewith the mechanism of GSH action can be dual. On the one hand, it neutralizes ROS by acting directly as a trap for

free radicals, or by ensuring the work of the specific peroxidases. On the other hand, GSH restores several oxidized proteins, thus restoring the functional activity of the wide range of enzymes, receptors, and transcription factors, and contributing, in this way, to the rapid formation of compensatory-adaptive responses [48]. Our results showed that the use of C_{60} fullerene enhances the synthesis of glutathione on 6th and 7th days of the experiment (−59%, $p<0.05$). Thus, long-term use of C_{60} fullerenes promotes the faster and more efficient formation of the adaptation processes under conditions of electrical stimulation of skeletal muscle of rats.

In summary, in the process of muscle fatigue, metabolism is disturbed, and products of incomplete oxidation of oxygen, peroxides, and free radicals are formed. The use of C_{60} fullerenes, as powerful antioxidants, helps reduce oxidative processes in skeletal muscles by maintaining a balance between pro-oxidants and the antioxidant defense system.

4. Conclusion

Thus, these data indicate that after 5-day use of the studied agent C_{60} fullerene has a 50%–70% stronger effect on the resumption of muscle biomechanics after fatigue than NAC. It was found that after cessation of NAC injections, the value of integrated muscle power already on the first day did not differ significantly from the control (5%–7%), and on the second day, did not differ significantly from the control. At the same time, after cessation of C_{60} fullerene injections, its residual effect was 45%–50% on the first day, and on the second day, 17%–23% of the control.

There is a clear tendency for all the described biochemical parameters to decrease by about 15% with therapeutic administration of NAC and by 30%–40% with C_{60} fullerene after 5 days of their application. The significant difference (more than 25%) between the pro- and antioxidant balance parameters in the studied muscles and blood plasma of rats after C_{60}fullerene administration probably plays a key role in its long-term residual effect compared with NAC. This indicates prolonged kinetics of water-soluble C_{60}fullerene excretion from the body, which contributes to a long (at least 2 days) compensatory activation of the endogenous antioxidant system by C_{60} fullerene in response to muscle stimulation, which should be considered in the development of new therapeutic agents based on this powerful nanoantioxidant [49].

Data Availability

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

Ethical Approval

The study protocol was approved by the Bioethics Committee of Taras Shevchenko National University of Kyiv by 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 by the Law of Ukraine No. 3447–IV 21.02.2006, Kyiv, on the Protection of Animals from Cruelty during medical and biological research.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Biomechanical analysis was performed by Dmytro Nozdrenko and Kateryna Bogutska; biochemical analysis was performed by Olga Gonchar and Svitlana Prylutska; preparation and characterization of the samples were done by Yuriy Prylutskyy, Eric Täuscher, and Uwe Ritter; coordination of the research work, analysis of the data, and preparation of the manuscript were done by Yuriy Prylutskyy, Peter Scharff, and Uwe Ritter.

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МІНІСТЕРСТВО ОСВІТИ І НАУКИ УКРАЇНИ

КИЇВСЬКИЙ НАЦІОНАЛЬНИЙ УНІВЕРСИТЕТ ІМЕНІ ТАРАСА ШЕВЧЕНКА

НОЗДРЕНКО ДМИТРО МИКОЛАЙОВИЧ

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ДИСЕРТАЦІЯ

Механокінетика скорочення скелетних м'язів за експериментальних патологій та дії вуглецевих наночастинок

03.00.02 - біофізика

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

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