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RESEARCH ARTICLE

Cell-free DNA kinetics in response to muscle-damaging exercise: A drop jump study

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Abstract

A significant increase in circulating cell-free DNA (cfDNA) occurs with physical exercise, which depends on the type of exertion and the duration. The aims of this study were as follows: (1) to investigate the time course of cfDNA and conventional markers of muscle damage from immediately after to 96 h after muscle-damaging exercise; and (2) to investigate the relationship between cfDNA and indicators of primary (low-frequency fatigue and maximal voluntary isometric contraction) and secondary (creatine kinase and delayed-onset muscle soreness) muscle damage in young healthy males. Fourteen participants (age, 22 ± 2 years; weight, 84.4 ± 11.2 kg; height, 184.0 ± 7.4 cm) performed 50 intermittent drop jumps at 20 s intervals. We measured cfDNA and creatine kinase concentrations, maximal voluntary isometric contraction torque, low-frequency fatigue and delayed-onset muscle soreness before and at several time points up to 96 h after exercise. Plasma cfDNA levels increased from immediately postexercise until 72 h postexercise ($P < 0.01$). Elevation of post-exercise cfDNA was correlated with both more pronounced low-frequency fatigue ($r = -0.52$, $P = 3.4 \times 10^{-11}$) and delayed-onset muscle soreness ($r = 0.32$, $P = 0.00019$). Levels of cfDNA change in response to severe primary and secondary muscle damage after exercise. Levels of cfDNA exhibit a stronger correlation with variables related to primary muscle damage than to secondary muscle damage, suggesting that cfDNA is a more sensitive marker of acute loss of muscle function than of secondary inflammation or damaged muscle fibres.

KEYWORDS

blood markers, cell-free DNA, eccentric exercise, muscle damage

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1 INTRODUCTION

Performing unaccustomed exercise can lead to muscle damage, especially when muscles perform eccentric contractions (Chalchat et al., 2022). In contrast to concentric or isometric contractions, eccentric lengthening contractions cause more profound sarcomere damage that leads to a more severe inflammatory response (Markus et al., 2021). Drop jumps (DJs) are a very common plyometric exercise used in sports training that are eccentrically biased. They are also one of the most powerful tools for the examination of exercise-induced muscle damage (Dias et al., 2022; Tanabe et al., 2022). Mechanically demanding contractions, particularly those in muscles unaccustomed to eccentric contractions (Dargeviciute et al., 2013; Newham et al., 1987), such as those used in DJs, can result in disruption of muscle-fibre structure, protein leakage, prolonged force depression, delayed-onset muscle soreness (DOMS) and inflammation (Clarkson & Hubal, 2002; Peake et al., 2017). Several markers describe these changes in sports science; however, the mechanisms by which they function or are released are highly distinct.

Increases in plasma biomarkers, such as creatine kinase (CK), C-reactive protein or myoglobin, are often used to evaluate the magnitude of exercise-induced muscle damage (Dias et al., 2022; Dupuy et al., 2018). However, there has been much debate in the last few years about the use of CK, e.g., owing to its slow reaction time and declining response to increased training levels, which, in turn, decrease its sensitivity to changes in training load (Haller et al., 2023; Saw et al., 2016). Previous studies have suggested reduced muscle force after eccentric exercise as the most appropriate indirect marker (Damas et al., 2016; Paulsen et al., 2012). However, the relationship between levels of circulating muscle-specific proteins (myoglobin and CK) and muscle function appears to be inconsistent (Friden & Lieber, 2001). As an example, loss of force and CK activity have different time courses. Muanjai et al. (2019) reported that a decrease in force and a rise in CK levels represent different types of muscle damage, termed primary and secondary, respectively. Low-frequency fatigue (LFF; the ratio of forces generated at 20 and 100 Hz stimulation), which is attributable to decreased sarcoplasmic reticulum Ca^{2+} release and/or diminished myofibrillar Ca^{2+} sensitivity, in addition to an increase in reactive oxygen species, can also be used as an indirect marker of primary damage (Wyckelsma et al., 2020). Secondary damage results in structural changes to the muscle fibre. Furthermore, damage might be attributable to white blood cell infiltration (mainly neutrophil granulocytes) of the muscle or increased intracellular Ca^{2+} activating phospholipase and protease enzymes (Yamada et al., 2018). Neither of the discussed markers (CK or LFF) is sensitive enough to respond to both acute exercise and secondary muscle damage, making alternative biomarkers necessary.

Cell-free DNA (cfDNA), which is DNA released from cells that circulate in the bloodstream, could potentially serve as a substitute marker for exercise-induced muscle damage (Andreatta et al., 2018). Cell-free DNA is known to be a pro-inflammatory load-dependent marker of aerobic and anaerobic exercise (Breitbach et al., 2012; Huminska-Lisowska et al., 2021). An increase in the level of this marker is associated with the activation and recruitment of immune cells (Korabecna et al., 2020). In healthy individuals, exercise is the primary external variable contributing to increased cfDNA levels (Andreatta et al., 2018; Atamaniuk et al., 2010; Haller et al., 2017). Many of the published studies have used protocols that require muscles to perform concentric and eccentric contractions, such as cycling (Tug et al., 2017), aerobic running (Haller et al., 2017), marathon (Atamaniuk et al., 2008) or lifting exercise (Atamaniuk et al., 2010), and light and heavy resistance training (Andreatta et al., 2018), meaning that simultaneous metabolic stress (accumulation of metabolites, i.e., lactate, H^+ ions in muscle cells and altered calcium homeostasis) and mechanical stress (structural changes involving sarcomere, cytoskeletal and membrane damage; disruption of excitation–contraction coupling) would occur. Only one study focused solely on eccentric training, where high-intensity and low-intensity eccentric cycling exercise was compared (Mavropalias et al., 2021). Although the protocol involved eccentric exercise, the participants performed high-intensity cycling, in which the accumulation of metabolites would have been present but lower than that of exercise in most previous studies (Juškevičiūtė et al., 2023; Tug et al., 2017). Hence, the extent to which the increase in cfDNA is caused by mechanically demanding exercise alone is unclear. To our knowledge, no studies have investigated the effects of DJs on cfDNA concentrations. In the present study, we wanted to observe how mechanically demanding exercise, without placing the organism under metabolic stress, affects cfDNA levels. It was thought that muscle contraction without the accumulation of metabolites, such as lactate, could increase cfDNA levels, first, because of physical exertion and, second, because of neutrophil infiltration into tissues owing to

Highlights

- **What is the central question of this study?**
  How do plasma cell-free DNA (cfDNA) levels change in response to primary and secondary muscle damage when muscle-damaging exercise is performed without metabolic stress?
- **What is the main finding and its importance?**
  In this study, we found that cfDNA concentration increases following severe primary and secondary muscle damage after 50 drop jumps. However, acute postexercise increases in cfDNA were higher than following secondary muscle damage and were more correlated with variables related to primary muscle damage (low-frequency fatigue). This suggests that muscle-damaging exercise causing no accumulation of metabolites can lead to acute and delayed increases in cfDNA levels. However, they are more sensitive to primary muscle damage.

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local inflammation and oxidative stress (Mortaz et al., 2018), because neutrophils are essential in the release of cfDNA (Moss et al., 2018).

Here, we aimed to examine the time course of plasma cfDNA levels after intermittent DJs and assess the extent to which cfDNA increases in the absence of metabolic stress. We also analysed whether plasma cfDNA levels change in response to DOMS and levels of CK, and whether there are associations between plasma cfDNA levels and neuromuscular fatigue indices. We aimed to test the hypothesis that cfDNA reacts to acute exercise (primary damage), in addition to delayed muscle damage (secondary damage).

2 | MATERIALS AND METHODS

2.1 | Ethical approval

The trial procedures were authorized by the Kaunas Region Biomedical Research Ethics Committee (no. BE-2-35) and the Human Ethics Committee Rhineland-Palatinate (no. 2019-14256), and all experiments were performed in accordance with the *Declaration of Helsinki*. All participants were informed orally and in writing about the experimental protocol and the aim of the study and gave written agreement to participate.

2.2 | Participants and familiarization

Healthy male participants were recruited (mean ± SD; age, 22 ± 2 years; weight, 84.4 ± 11.2 kg; height, 184.0 ± 7.4 cm; $n = 14$) who did not participate in any official exercise or sports programme but were physically active by the standard of participating in recreational physical activity two or three times per week. Participants were asked to abstain from any strength and conditioning programme for 72 h before the pretest and during the test week. Exclusion criteria for participation in the study included individuals who currently take any medications or suffer from neuromuscular disease or cardiovascular disease. Three days before the initial study, each participant completed a familiarization session to become familiar with all the measurements. During the same visit, tolerance to electrical stimulation was assessed. As a precaution, participants performed only one or two DJs for learning purposes before the trial to avoid muscle damage.

2.3 | Drop jumps

Every participant performed 50 DJs at intervals of one every 20 s. The interval of 20 s was chosen to minimize metabolic stress. Fifty DJs were chosen because they induce sufficient primary and secondary damage (Skurvydas et al., 2011).

Each subject underwent exercise testing between 08.00 and 10.00 h. Upon arrival at the laboratory, subjects were asked to sit calmly for 10 min in the room. Blood samples were taken, then subjects performed an 8–10 min warm-up on an electrically braked cycle ergometer (Ergo-Fit, Pirmasens, Germany) with a pedalling frequency that ranged between 60 and 70 r.p.m. After warming up, the participants underwent neuromuscular tests, and blood samples were collected again (pre-exercise). After that, the DJs were conducted (they were performed only once, on the first day of the study). Neuromuscular tests and blood collection were repeated immediately after exercise and at +45 min, +90 min, +6 h and +12 h postexercise and on the following days (24, 48, 72 and 96 h postexercise) (Figure 1a). Subjects performed DJs from a height of 0.5 m to a 90° knee angle, with immediate maximal vertical rebound and with 20 s rest between each DJ. During the experiment, an experienced investigator controlled the knee angle visually. Each subject placed their hands on their hips to isolate the contribution from the upper limbs. The subjects were verbally encouraged to jump as high as they could. Participants stepped up to the platform with their left leg, and the force of muscle contraction in the right leg was tested. A contact mat (Newtest Powertimer Testing system, Oulu, Finland) was used for performing the DJs. For the next 4 days, participants underwent neuromuscular tests as described below, and blood samples were taken.

2.4 | Neuromuscular testing

Neuromuscular testing, consisting of electrically evoked torque and maximal voluntary isometric contraction (MVIC) of the knee extensors, is illustrated in Figure 1b. The participants sat upright on the seat of an isokinetic dynamometer (System 4; Biodex Medical Systems, Shirley, NY, USA) calibrated according to the manufacturer's recommendations. Belts were attached to the shank, trunk and shoulders to stabilize them. The dynamometer was set with the knee joint positioned at an angle of 90° (180° = full extension) during MVIC and electrical stimulations (Treigyte et al., 2024).

Electrical stimulations were applied using three 12 cm × 8 cm carbonized rubber surface electrodes (MARP Electronic), lubricated with electrode gel (EGG-EEG Gel, Modi'in, Israel). Two electrodes were positioned vertically and transversely across the width of the proximal portion of quadriceps muscles, and the third one covered the distal portion of the quadriceps muscles above the patella (Figure 1c). To ensure similar electrode placement during each measurement, the electrode sites were marked with a permanent marker. In between experiments, participants were instructed not to erase these marks. An electrical stimulator (Digitimer DS7A; Digitimer, Welwyn Garden City, UK) was connected to the electrodes to deliver 0.5 ms square-wave pulses at a constant current set at 100 mA and constant voltage limit set at 200 V (Eimantas et al., 2022).

Neuromuscular testing began with two electrical stimulations separated by 3 s of rest: 1 s stimulation at 20 Hz (P20) and 1 s stimulation at 100 Hz (P100). Peak torques were determined for these electrical stimulations, and P20/P100 was calculated. Directly after these stimulations, two MVICs of the knee extensor muscles were performed and separated by a rest period of 1 min. The MVIC torque corresponded to the maximum torque after 2–3 s. The participants were verbally encouraged to exert and maintain maximal effort for

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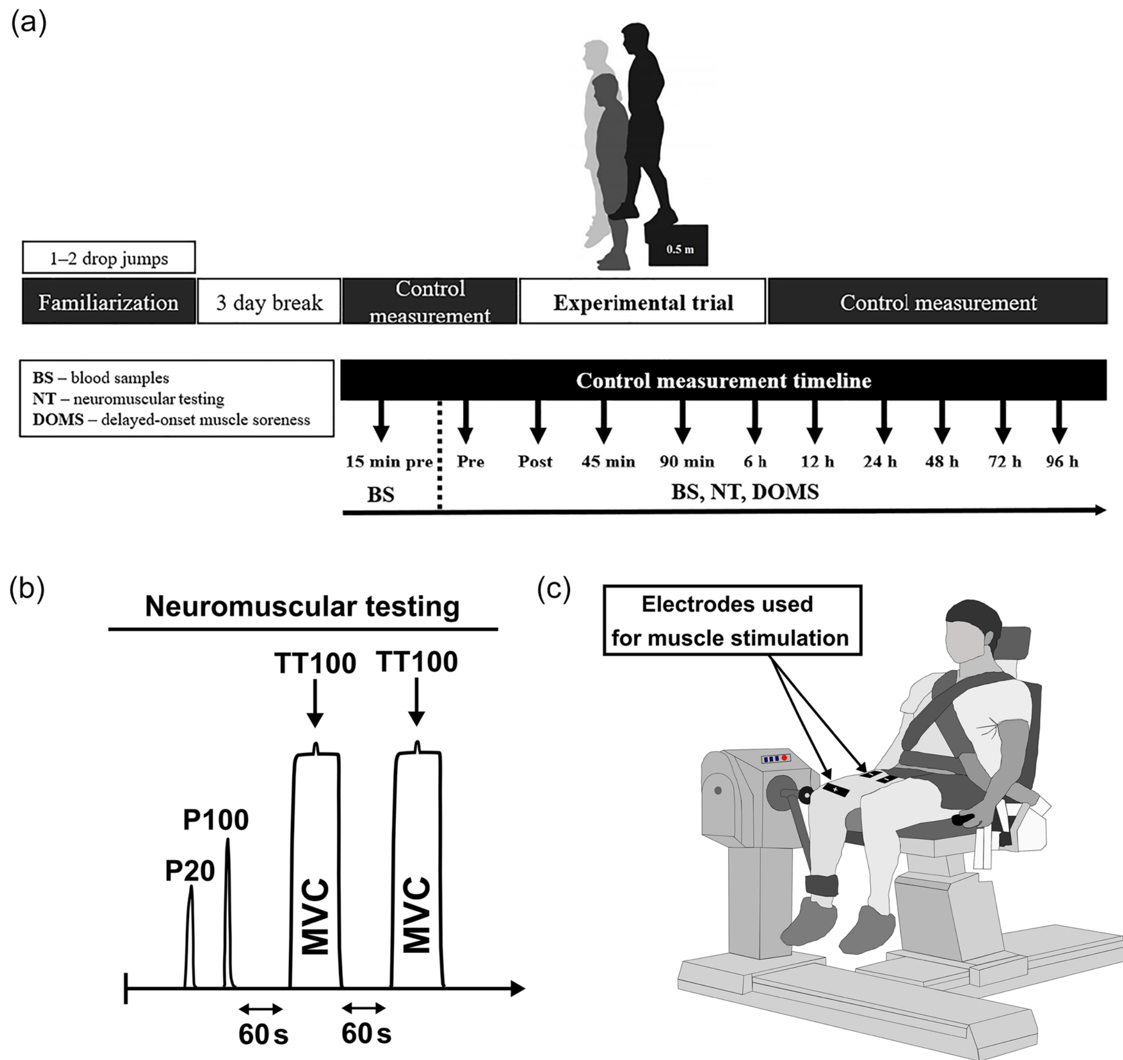

FIGURE 1 (a) Experimental design. (b) Neuromuscular testing. (c) Position of electrodes placed on the quadriceps. Abbreviations: BS, blood samples; DOMS, delayed-onset muscle soreness; MVIC, maximal voluntary isometric contraction; NT, neuromuscular testing; P20, electrical stimulation (1 s stimulation) at 20 Hz; P100, electrical stimulation (1 s stimulation) at 100 Hz; TT, 250 ms test train stimulation at 100 Hz.

~5 s, and a 250 ms test train stimulation at 100 Hz was superimposed on voluntary contraction 3–4 s into the MVIC. The central activation ratio (CAR), a measure of voluntary activation level, was calculated as (MVIC torque/total peak torque generated with the superimposed 250 ms test train stimulation) × 100 (Treigyte et al., 2024). The highest MVIC torque value from two attempts was used for further analysis.

2.5 | Muscle soreness

Participants subjectively assessed muscle soreness (DOMS) using a visual scale of 0–10, where 0 represented no pain and 10 indicated intolerably intense pain (Lau et al., 2015). Participants were requested to evaluate the severity of soreness in their exercised quadriceps during two or three squats (Skurvydas et al., 2011). Muscle soreness was assessed pre- and postexercise and at +45 min, +90 min, +6 h, +12 h, +24 h, +48 h, +72 h and +96 h after the DJs.

2.6 | Blood sampling and processing

Fifteen minutes and immediately before exercise, immediately post-exercise and at +45 min, +90 min, +6 h, +12 h, +24 h, +48 h, +72 h and +96 h after exercise, a 9 mL sample of venous blood was collected in EDTA-collection tubes (EDTA Monovettes; Sarstedt, Germany) and were immediately centrifuged at 1600*g* and 4°C for 10 min. Plasma was transferred to a fresh tube, followed by a second 10 min of centrifugation at 16,000*g* and 4°C. Aliquots were stored at −20°C until further analysis.

2.7 | Plasma creatine kinase and blood lactate

Blood lactate concentration was measured before exercise, immediately after exercise and at 45 min postexercise using an *Accutrend* portable lactate analyser (Roche, Germany). Creatine kinase

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activity was measured using an automatic biochemical analyser (Spotchem EZ SP-4430; Arkray, Kyoto, Japan) at the 11 time points described above.

2.8 | Quantification of cfDNA

Concentrations of venous cfDNA were quantified by analysing unpurified plasma via quantitative real-time (qPCR). Direct qPCR is based on the amplification of one length of an abundant L1PA2 repeat, which is a subfamily of the human long interspersed element of class 1. L1PA2 sequences are distributed over all chromosomes and constitute almost 17% of the human genome (Beck et al., 2010). In brief, diluted plasma (1:10 in H_2O) was used as a template for qPCR. The amplification was based on primers targeting a 90 bp fragment (5′-TGCCGCAATAAACATACGTG-3′ and 5′-GACCCAGCCATCCCATTAC-3′) of human long interspersed nuclear elements of the L1PA2 family. Samples were analysed with a CFX384 Touch Real-Time PCR system (Bio-Rad, München, Germany) using the following protocol: 2 min incubation at 98°C, followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 64°C for 40 s and extension at 75°C for 10 s (Neuberger et al., 2022). All samples were run in triplicate. If the triplicates of the samples showed an SD of the quantification cycle (*Cq*) > 0.4, native plasma samples were rediluted and reanalysed.

2.9 | Data analysis

The qPCR data were captured using the CFX Manager Software, v.3.1 (Bio-Rad, Hercules, CA, USA) and Microsoft Excel, 2016. Statistical analysis was performed using R software, v.4.0.3. Data are presented as the mean ± SD. The assumption of normality was assessed using the Shapiro–Wilk normality test. A one-way repeated-measures ANOVA was used to assess differences between time points. A significant test was followed by *post hoc* tests or Wilcoxon rank-sum tests, for normally and non-normally distributed data, respectively. A Bonferroni–Holm adjustment method was used for *P*-value correction. Correlations between normally distributed and non-normally distributed data were investigated using Pearson and Spearman correlation tests. The ggplot2 package v.3.2.2 was used for graphical illustrations. We considered *P*-values < 0.05 to be statistically significant.

3 | RESULTS

All 14 participants accomplished 50 DJs. Neuromuscular testing was completed, and blood samples and subjective reports of muscle soreness were collected before and after exercise and for the ensuing 4 days.

The height of DJs from the first DJ to the last DJ was not significantly different (30.37 ± 5.55 and 36.39 ± 6.83 cm, respectively, $P > 0.05$).

3.1 | Voluntary and electrically evoked muscle torque

Data for P20, P100, MVIC torques, CAR and LFF were expressed relative to baseline values set to 100% in each experiment. The within one-way repeated-measures ANOVA showed that the effects of time ($P < 0.01$) on P20, P100 and MVIC were significant (Figure 2). The MVIC torques were decreased to 83.1% ± 5.75% directly after exercise ($P < 0.001$) and were progressively restored to the baseline values over the recovery period (time effect: $P < 0.001$; Figure 2a). There was a slight decrease in CAR immediately after exercise (94.1% ± 5.76%, $P < 0.05$), 45 min postexercise (93.8% ± 7.21%, $P < 0.05$) and 90 min postexercise (90.7% ± 8.77%, $P < 0.05$), but it was restored after 6 h ($P > 0.05$) (Figure 2d).

The P20 and P100 torques were significantly affected by the experimental exercise (Figure 2b, c). The P20 torque was significantly decreased postexercise (49.7% ± 17.8%, $P < 0.001$) compared with the pre-exercise value (100%) and recovered only at 72 h after exercise (92.5% ± 15.0%, $P > 0.05$). As shown in Figure 2c, the P100 torque was decreased because of DJs (79.5% ± 15.4%, $P < 0.01$), but the P100 torque was restored at 12 h after exercise (91.9% ± 10.6%, $P > 0.05$). The decrease in the P20/P100 ratio showed that LFF exists after 50 DJs (61.3% ± 15.3%, $P < 0.0001$) (Figure 3). After 48 h of recovery, LFF was not fully recovered but was not significantly different from the pre-exercise values (88.4% ± 13.0%, $P > 0.05$).

3.2 | Delayed-onset muscle soreness and CK activity

Plasma CK activity increased after DJ exercise (time effect: $P < 0.001$) (Figure 4a). A significant increase was found, from 2.22 ± 0.29 to 2.31 ± 0.24 IU/L on a $\log_{10}$ scale at 90 min postexercise ($P < 0.05$), to 2.62 ± 0.26 IU/L on a $\log_{10}$ scale at 6 h postexercise ($P < 0.001$), and CK values peaked at 12 h postexercise (2.77 ± 0.28 IU/L on a $\log_{10}$ scale, $P < 0.001$). A significant effect of time was found for muscle soreness (time effect: $P < 0.001$), which peaked at 1–2 days postexercise (Figure 4b). At time points 24 and 48 h postexercise, all subjects reported having DOMS symptoms (4.31 ± 1.65 at 24 h post-exercise, $P < 0.05$; 4.31 ± 2.50 at 48 h postexercise, $P < 0.05$). Muscle soreness (1–2 on a scale from 0 to 10) was reported by six participants at time point 96 h (1.77 ± 1.79), but it was not statistically significant compared with the pre-exercise value ($P > 0.05$).

3.3 | Lactate

We found a significant decrease in lactate concentration immediately postexercise (from 1.52 ± 0.29 to 1.21 ± 0.35 mmol/L, $P = 0.016$), and the decrease was still evident 45 min postexercise (1.2 ± 0.28 mmol/L, $P = 0.012$).

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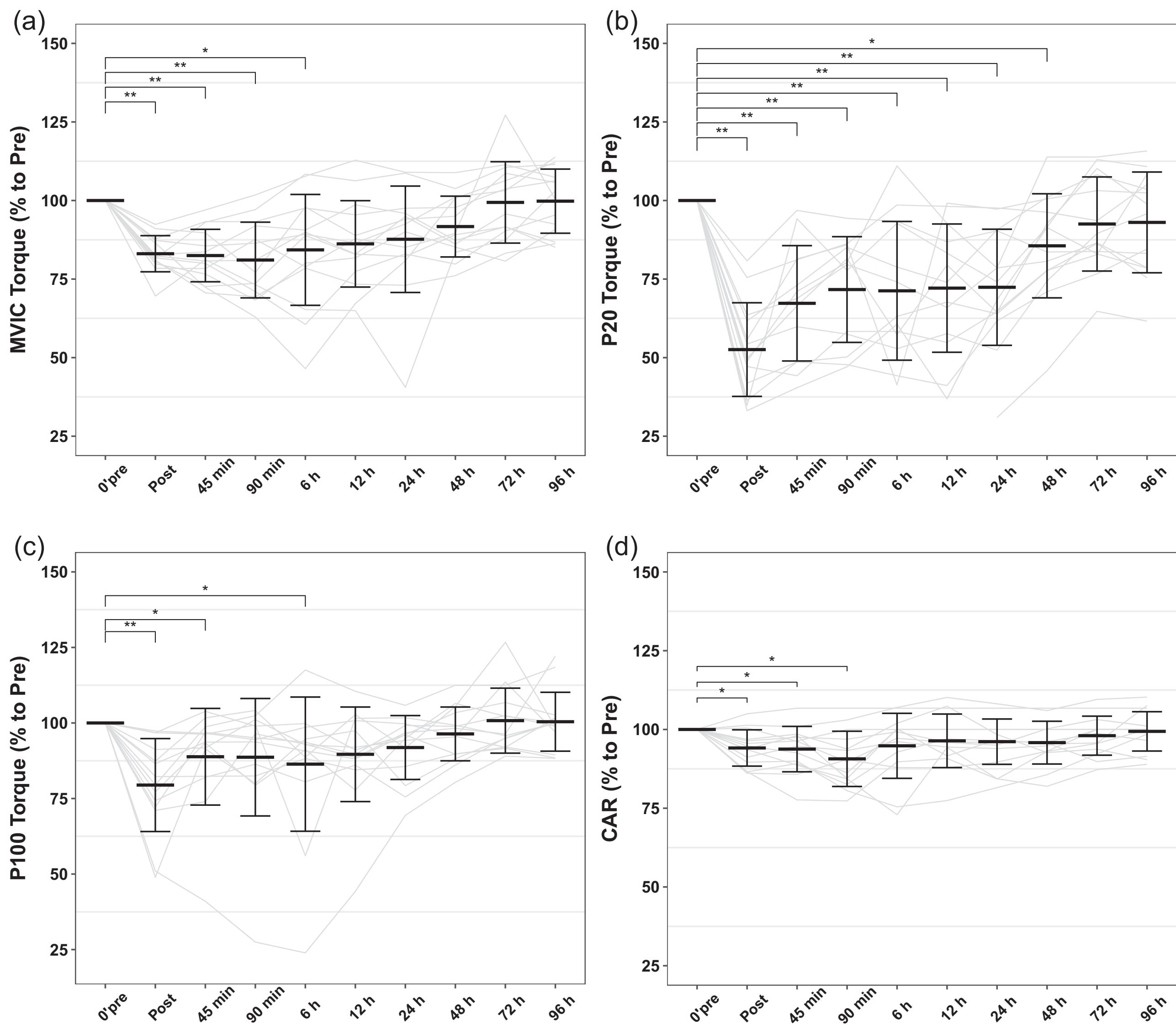

FIGURE 2 (a) MVC torque. (b) P20 torque. (c) P100 torque. (d) CAR. Data are expressed relative to baseline values, which were set to 100% in each experiment. Data are shown as means ± SD and individual values, $n = 14$. *$P < 0.05$, **$P < 0.01$: differences from the Pre time point. Abbreviations: CAR, central activation ratio; MVIC, maximal voluntary isometric contraction; P20, 1 s stimulation at 20 Hz; P100, 1 s stimulation at 100 Hz.

3.4 | Plasma cfDNA concentration

Cell-free DNA (90 bp) values increased acutely after 50 DJs (from 0.97 ± 0.19 to 1.5 ± 0.21 ng/mL on a $\log_{10}$ scale, $P < 0.01$) and normalized 90 min postexercise (1.09 ± 0.3 ng/mL on a $\log_{10}$ scale, $P > 0.05$) (Figure 5). A significant increase in the cfDNA concentration was also noticed after 6 h (1.25 ± 0.25 ng/mL, $P < 0.01$), 12 h (1.29 ± 0.27 ng/mL, $P < 0.01$), 48 h (1.26 ± 0.41 ng/mL, $P < 0.05$) and 72 h (1.42 ± 0.55 ng/mL, $P < 0.01$). At 96 h after exercise, cfDNA values had recovered and did not differ significantly from pre-exercise levels ($P > 0.05$).

3.5 | Correlations

Cell-free DNA levels were significantly correlated with LFF ($r = -0.52$, $P = 3.4 \times 10^{-11}$) (Figure 6a). The correlation was negative, indicating that greater increases in cfDNA were associated with a more pronounced LFF. Likewise, there was an association between cfDNA concentrations and P20 torque ($r = -0.45$, $P = 4.8 \times 10^{-8}$) (Figure 6c). Additionally, cfDNA levels were significantly correlated with P100 torque ($r = -0.3$, $P = 0.00038$) and MVIC torque ($r = -0.29$, $P = 0.00048$) (Figure 6b, d). We found no correlation with CK over all time points ($r = -0.061$, $P = 0.47$) (Figure 6e). However, there

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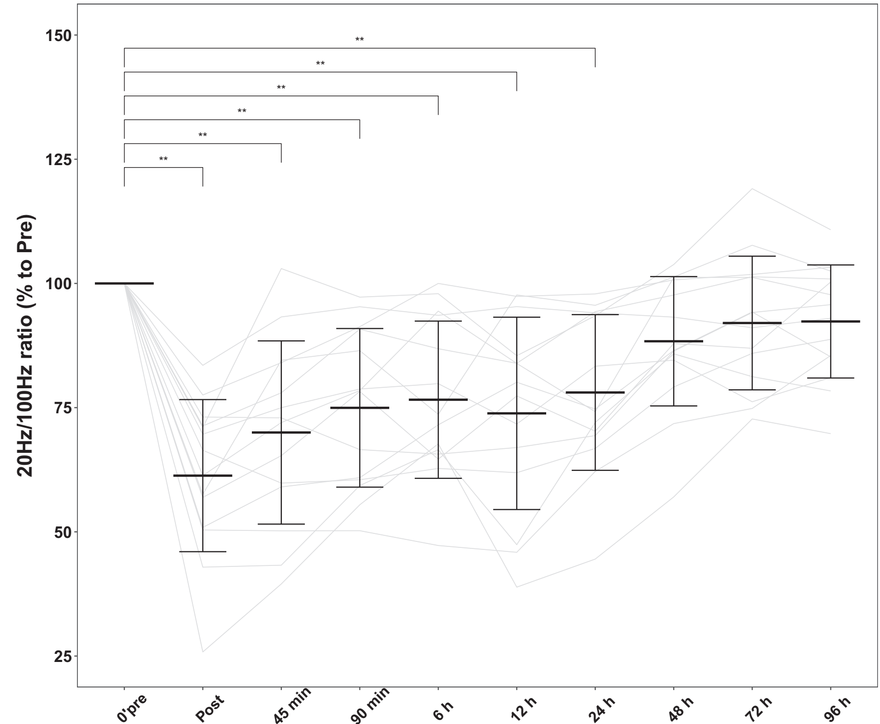

FIGURE 3 Low-frequency fatigue (ratio of 20 Hz to 100 Hz electrically stimulated torques). Data are expressed relative to baseline values, which were set to 100% in each experiment. Data are shown as means ± SD and individual values, $n = 14$. $^{**}P < 0.01$: differences from the Pre time point.

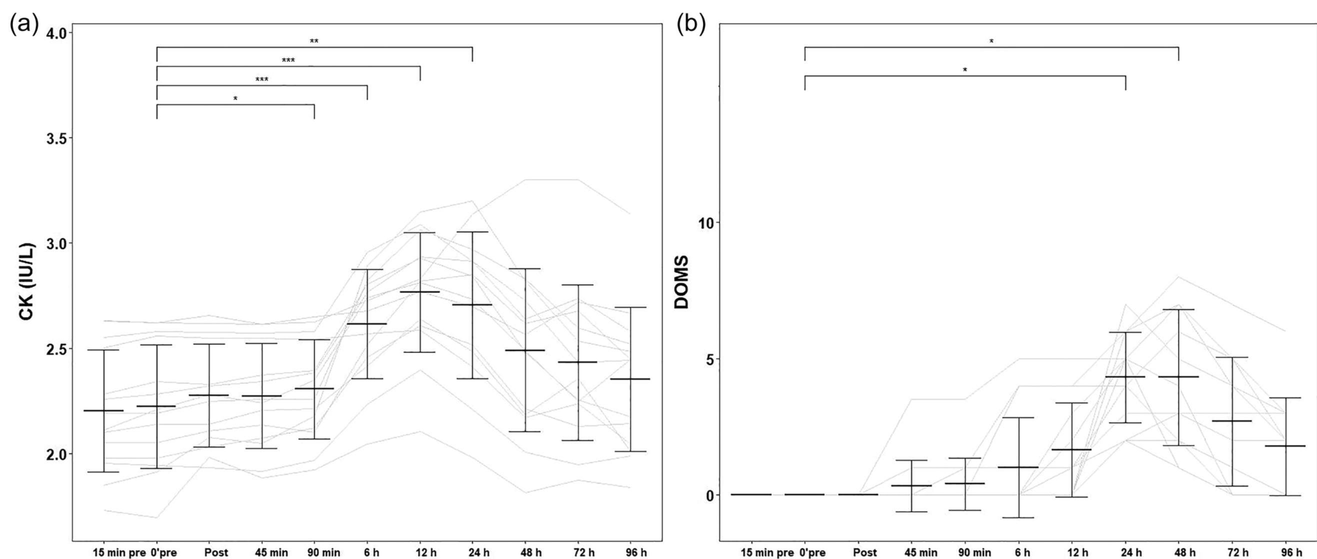

FIGURE 4 (a) CK. (b) DOMS (0–10 scale). Data are shown as mean ± SD and individual values, $n = 14$. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$: differences from the Pre time point. Abbreviations: CK, creatine kinase; DOMS, delayed-onset muscle soreness.

was a moderate correlation between cfDNA (90 bp) levels and DOMS ($r = 0.32$, $P = 0.00019$) (Figure 6f), where higher increases in cfDNA were associated with a greater degree of muscle soreness.

4 | DISCUSSION

The effect of muscle-damaging exercise without metabolic stress on cfDNA levels is unclear. Most studies have included exercises that are, for the most part, metabolically demanding (high metabolic stress), suggesting that this research is crucial in the context of cfDNA. Subjects performed repeated DJs at long (20 s) intervals, an exercise that has been shown to induce severe mechanical stress, whereas long intervals prevent any major metabolic challenges (Kamandulis et al., 2017). Furthermore, DJs are short intervals of work (~1–2 s for a DJ), meaning that participants trained for a very short period (~1–2 min). What has not been examined previously in any detail is the change in cfDNA kinetics after eccentric exercise without metabolic

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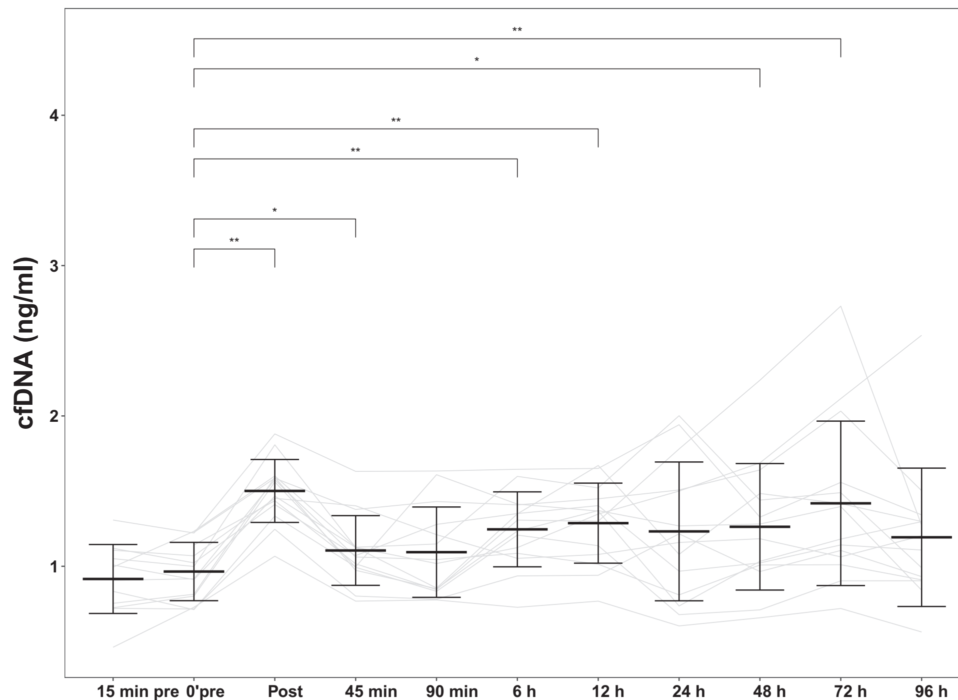

FIGURE 5 Cell-free DNA (90 bp) kinetics over the assessment period. Data are shown as means ± SD on a logarithmic scale, $n = 14$. *$P < 0.05$ and **$P < 0.01$: differences from the Pre time point. Abbreviation: cfDNA, cell-free DNA.

stress. Nor has the relationship been examined between cfDNA levels (postexercise or at 1, 2 or 3 days after exercise) and DOMS, CK and neuromuscular function (MVIC torque and LFF) following this type of exercise. Overall, the present study suggests that 50 DJs can lead to distinct time-course changes in cfDNA levels. In comparison to other measured variables (CK, DOMS, MVIC and LFF) that were altered owing to either severe primary or secondary damage, only cfDNA changed in the presence of these two events as a result of 50 DJs. In addition, the magnitude of the increase in cfDNA was significantly correlated with LFF, MVIC and DOMS. Nevertheless, whether and how these changes depend on the DJ dosage must be explored.

Our study shows that 50 DJs performed at long intervals did not cause an accumulation of metabolites. Lactate did not increase after exercise, even though the exercise included repetitive eccentric contractions that induce mechanical stress. Other studies have shown that lactate levels decrease after exercise, suggesting accelerated lactate uptake by the muscles (Spengler et al., 1999). There, lactate is used as a substrate for energy production through oxidative phosphorylation and by enhanced lactate transport by monocarboxylate transporters on the surface of muscle cells (Gladden, 2004).

In the present study, the rise in plasma cfDNA occurred immediately postexercise and after 6, 12, 48 and 72 h, possibly reflecting primary damage (first increase) and local inflammation (second increase). Our results of moderate increases in cfDNA levels (~5-fold) were higher than the increases in cfDNA levels measured by Atamaniuk et al. (2010) after a single bout of high-intensity strength exercise (six sets of six lifting exercises with 90%–95% of one-repetition maximum) (3.3-fold) but lower than the increases of cfDNA levels measured by Beiter et al. (2011) after exhaustive short-term treadmill exercise (9.9-fold). It has been reported that cfDNA levels depend on exercise modality, intensity and duration and are more pronounced after long-lasting highly metabolically demanding exercise than after short low-intensity exercise (Neuberger et al., 2022; Tug et al., 2017). The findings in this study show that only 1–2 min of mechanically demanding exercise, with no metabolic stress, can significantly increase cfDNA levels. In the present study, the initial elevation of cfDNA concentration might be linked to an aseptic inflammatory response (Tug et al., 2017) and caused by mechanical stress (Markus et al., 2021). If so, circulating cfDNA is released as an active secretion, and its levels immediately postexercise will be derived mainly from haematopoietic cells (Moss et al., 2018; Neuberger et al., 2022). An active release, which involves spontaneous release of cfDNA in free or encapsulated form, is the main mechanism of cfDNA release after acute exercise (Hu et al., 2021; Neuberger & Simon, 2022). Cell-free DNA released by cell injury or death pathways, such as apoptosis or necrosis, refers to passive mechanisms (Grabuschnig et al., 2020; Han & Lo, 2021) and is usually a slow process. In the present study, the second increase in cfDNA levels at 6, 12, 48 and 72 h postexercise could be attributed to a local inflammatory reaction that happens through direct mechanical lesions of the muscle sarcomere (Brancaccio et al., 2010) and indirect formation of oxygen free radicals (Radak et al., 2008; Webb et al., 2017). Consequently, neutrophils infiltrate tissues owing to local inflammation and oxidative stress (Mortaz et al., 2018). Their role in releasing cfDNA is crucial (Moss et al., 2018; Neuberger et al., 2022) and thus might contribute to delayed elevation of the cfDNA level. It should be noted that we did not investigate the source of cells

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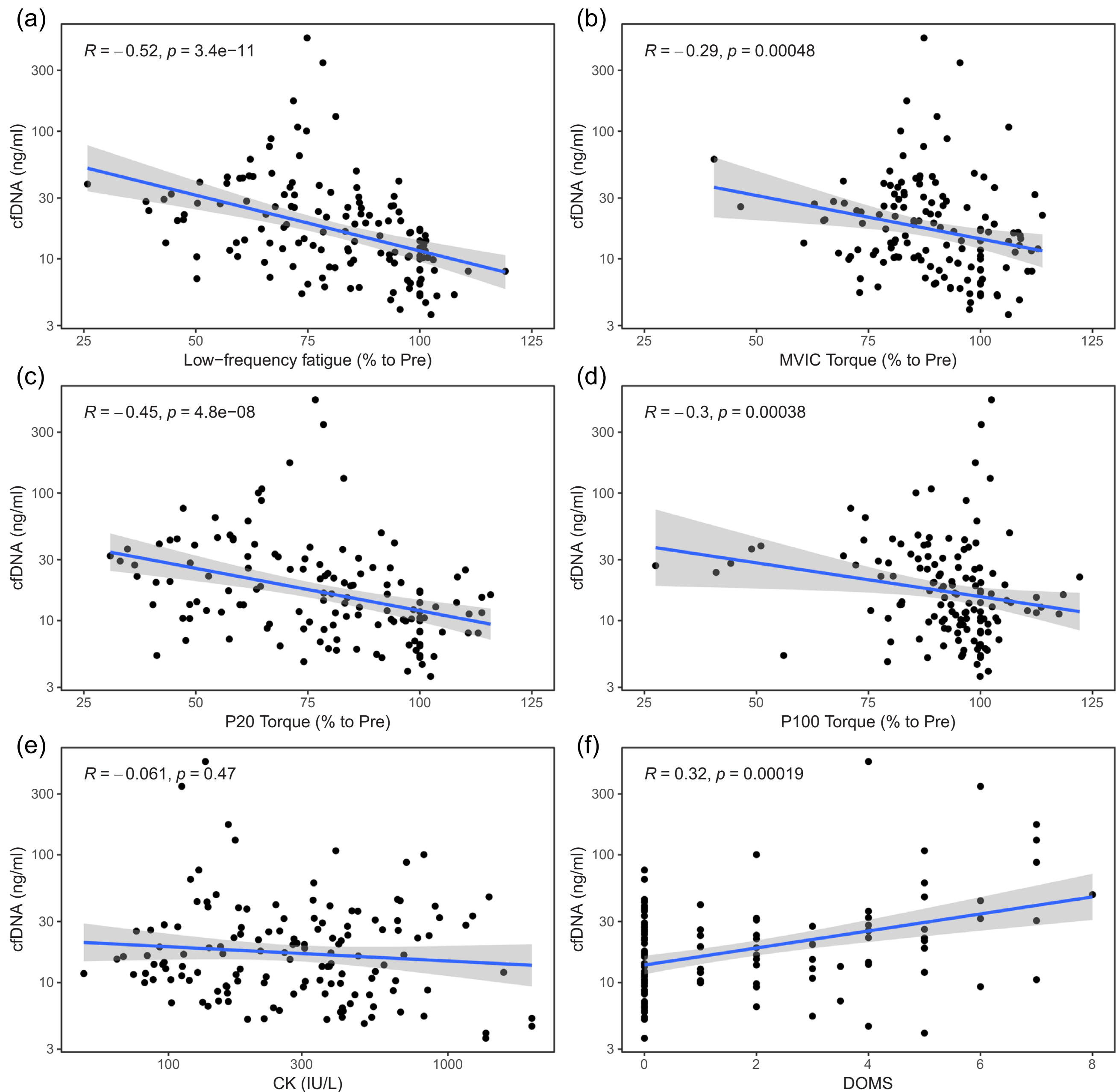

FIGURE 6 (a) Correlation between low-frequency fatigue (ratio of 20 Hz to 100 Hz electrically stimulated torques) and $\log_{10}$-transformed cfDNA, $n = 14$. (b) Correlation between MVIC torque and $\log_{10}$-transformed cfDNA. (c) Correlation between P20 torque and $\log_{10}$-transformed cfDNA, $n = 14$. (d) Correlation between P100 torque and $\log_{10}$-transformed cfDNA, $n = 14$. (e) Correlation between $\log_{10}$-transformed CK and $\log_{10}$-transformed cfDNA, $n = 14$. (f) Correlation between DOMS and $\log_{10}$-transformed cfDNA, $n = 14$. The lines represent the linear trend. The significance of the correlation and r values are displayed on the charts. Abbreviations: cfDNA, cell-free DNA; CK, creatine kinase; DOMS, delayed-onset muscle soreness; MVIC, maximal voluntary isometric contraction; P20, 1 s stimulation at 20 Hz; P100, 1 s stimulation at 100 Hz.

releasing cfDNA in this study. Further studies will be needed to identify the cells contributing to primary and secondary cfDNA release after muscle-damaging exercise.

Structural damage to the contractile apparatus and cell membrane leads to reduced muscle function and local sterile inflammation (Paulsen et al., 2010, 2012). A decrease in muscle force-generating capacity after eccentric exercise is a valuable tool as an indirect indicator of the severity of skeletal muscle damage. In the present study, MVIC was decreased immediately postexercise after 50 DJs. Muscle damage or fatigue can cause MVIC reductions soon after exercise (Souron et al., 2018). Given that CAR exhibited slight impairment throughout the assessment period, the decrease in MVIC torques following DJs might reflect muscular impairment rather than a decrease in neuronal activity (Kamandulis et al., 2022). Furthermore, LFF was significantly reduced immediately after exercise (~40% decrease from Pre) and did not recover during the next 48 h post-

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exercise. Similar to our results, Kamandulis et al. (2022) found an ∼45% reduction in LFF after 50 DJs. The underlying mechanisms of LFF differ depending on whether it is caused by metabolic or mechanical stress during exercise (Skurvydas et al., 2016). We recently reported a correlation between cfDNA levels and LFF during highly metabolically demanding exercise (Juškevičiūtė et al., 2023). The present study showed a stronger correlation between these two variables when only mechanical stress was applied, in comparison to our previous results on young subjects. Mechanically induced LFF is generally accepted to be caused by structural disturbances within muscle fibres (Kamandulis et al., 2017; Lauritzen et al., 2009), but impaired transmission between muscle fibres owing to distorted extracellular structural elements cannot be excluded (Kamandulis et al., 2022). A strong inflammatory response follows in response to these structural disturbances (Fatouros & Jamurtas, 2016), accompanied by an increase in neutrophil counts and other inflammatory cells, such as natural killer cells and lymphocytes (Stožer et al., 2020). Thus, neutrophil changes would contribute to increased cfDNA levels. Consequently, there is a correlation between elevated cfDNA levels and more pronounced LFF after exercise. Because the restoration of muscle function after exercise can be considered of clear practical value, the response of some additional variables, such as cfDNA, in the days following exercise should also be monitored for signs of improvement.

In addition, we wanted to determine whether the postexercise cfDNA concentration could be associated with secondary muscle damage markers, such as CK and DOMS. However, there was no significant correlation between the magnitude of the increase in plasma cfDNA concentration and CK. The elevation of CK concentration, which did not return to baseline values by the end of the experiment, in our study was similar to that observed by Kamandulis et al. (2022) following 50 DJs in healthy young male subjects. Studies show that there is often a poor relationship between functional outcomes and CK activity (Koch et al., 2014), as evidenced by the results of our study (no correlation was found between CK and LFF, $r = -0.04$, $P = 0.64$). In line with our results, Andreatta et al. (2018) showed a better correlation of cfDNA with muscle performance (they evaluated muscular performance by both squat jump and countermovement tests) than CK concentration after leg-press exercise with high and low intensity. However, in contrast to our findings, significantly increased lactate levels were found in their study (Andreatta et al., 2018).

Delayed muscle soreness is associated with the inflammatory response and muscle damage (Cheung et al., 2003), and we hypothesized that postexercise DOMS might be linked to elevated cfDNA levels. We found a significant correlation between cfDNA and DOMS. Studies have found several mechanisms associated with DOMS, including mechanical strains in muscle structures, overstretching sarcomeres, biochemical reactions that affect muscle contractile structures, and inflammatory processes related to oxidative stress and heightened sensitivity of pain receptors (da Silva et al., 2021). In previous studies, cfDNA has been associated with increased pain perception (Fleckenstein et al., 2021). There is also a link between cfDNA and pain in conditions such as sickle-cell disease (Al-humood et al., 2014) and *Bothrops* envenomation (de Souza Barbosa et al., 2021). As a physiological phenomenon, soreness occurs around extracellular structures, the fascia of the muscles and the nervous system (sensitization) (Paulsen & Benestad, 2019). More precisely, sensations of muscle soreness could result from a complex interaction of damage to the muscle structure, disrupted Ca^{2+} homeostasis or sensitization of nociceptors from inflammatory cell infiltrates (Hyldahl & Hubal, 2014). Sensitization of the peripheral and central nervous systems, including activation of nociceptors, is linked to the immune system and neuroinflammation (Starobova et al., 2020). In the early stages of inflammation, neutrophils, macrophages and mast cells can be recruited to relevant tissues, thereby initiating the maintenance and resolution of pain (Yang et al., 2022). Activation of an immune response modulates the excitability of pain pathways by forming an integrated network of immune cells, glia and neurons (Fleckenstein et al., 2021). Enhanced activation of immune cells, during and after exercise, might result in a more pronounced correlation between cfDNA and DOMS.

This study had limitations. Only young healthy men were included in this study, hence our results cannot be extrapolated to the general population, including females, older people and people with medical conditions. Moreover, there is controversy regarding sex-based differences in circulating cfDNA levels (Yuwono et al., 2021). Future studies should be expanded to include individuals of both sexes and all ages (Jylhava et al., 2011; Zhong et al., 2007).

AUTHOR CONTRIBUTIONS

Marius Brazaitis, Nerijus Eimantas and Ema Juškevičiūtė were responsible for the conception or design of the work and conduction of the experiments. Ema Juškevičiūtė, Marius Brazaitis, Kirsten Heinkel, Elmo Neuberger and Perikles Simon were responsible for analyzing and interpreting the data. All authors critically revised the work for important intellectual content. All authors have read and approved the manuscript and agree to be acountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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CONFLICT OF INTEREST

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DATA AVAILABILITY STATEMENT

The data that support the findings of the present study are available from the corresponding author on reasonable request.

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