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Dose-response of Muscle Damaging Exercise on Cell-free DNA

Ema Juškevičiūtė[1], Elmo Neuberger[2], Nerijus Eimantas[1], Perikles Simon[2], Marius Brazaitis[1]
[1]Institute of Sport Science and Innovations, Lithuanian Sports University, Kaunas, Lithuania
[2]Department of Sports Medicine, Disease Prevention and Rehabilitation, Johannes Gutenberg University Mainz, Mainz, Germany

ABSTRACT

Background: It has been observed that severe muscle damage induced by drop jumps has a significant effect on cell-free DNA (cfDNA) concentration. We hypothesized that a reduced volume of drop jumps would indicate a dose-dependent release of cfDNA.

Methods: Seven participants (aged 21 ± 1.5 years) performed 25, and 4 participants (aged 22 ± 1.8 years) performed 10, intermittent drop jumps (DJs) at 20 s intervals (DJ-25 and DJ-10 groups). We measured cfDNA, creatine kinase (CK), lactate concentrations and delayed-onset muscle soreness (DOMS) before and at several time points up to 96 h after exercise.

Results: There was a significant increase in plasma cfDNA levels immediately post-exercise in the DJ-25 group ($p = 0.012$). CK levels increased at 6, 12, 24 and 48 h post-exercise ($p = 0.003$, $p < 0.001$, $p < 0.001$, $p = 003$, accordingly) in the DJ-25 group. In the DJ-25 group only, DOMS values were increased at 12, 24 and 48 h post-exercise ($p < 0.05$).

Conclusion: cfDNA is responsive to muscle-damaging exercise in a dose-dependent manner, as only 25 DJs resulted in an immediate increase in cfDNA concentrations after exercise, while 10 DJs were insufficient to elicit any change.

Keywords: biomarker; eccentric exercise; muscle damage

INTRODUCTION

Eccentric biased exercise, where muscles are activated while lengthening, can improve athletic performance by increasing muscle mass, strength, and power (McNeill et al., 2019) and is useful as an injury prevention (Lastayo et al., 2014). However, eccentric lengthening contractions are more damaging to sarcomeres than concentric or isometric contractions, often resulting in muscle fiber damage, protein leakage, prolonged force depression, delayed onset muscle soreness (DOMS), and inflammation (Clarkson & Hubal, 2001; Peake et al., 2017). The extent of muscle damage is usually assessed by measuring various indirect markers, including creatine kinase (CK), oxidative stress markers, and myoglabin (Dupuy et al., 2018), or performing neuromuscular measures (Damas et al., 2016). While many markers describe these changes in sports science, their mechanisms of action and release are highly diverse. Muscle damage is divided into primary damage, which occurs due to decreased sarcoplasmic reticulum Ca2+ release and/or diminished myofibrillar Ca2+ sensitivity, as well as an increase in reactive oxygen species (ROS) (Wyckelsma et al., 2020), and secondary damage, which results in structural changes to the muscle fiber, as well as white blood cell infiltration of the muscle (Malm et al., 2000). However, neither of the mentioned markers is sensitive enough to respond to primary and secondary muscle damage. Recently, we (Juškevičiūtė et al., 2024) examined a rise in plasma cfDNA levels after muscle damaging exercise. To our knowledge this was the first study to demonstrate that muscle damaging exercise causing no accumulation of metabolites

can lead to acute and delayed increases in cfDNA levels (Juškevičiūtė et al., 2024). 50 intermittent drop jumps (DJs) at 20 s intervals induced severe primary and secondary muscle damage, yet we found a stronger correlation between cfDNA and variables related to primary muscle damage than to secondary muscle damage.

Circulating cfDNA refers to double-stranded DNA fragments found in body fluids such as plasma, serum, and urine, which arise from biological fragmentation due to stress (Humińska-Lisowska et al., 2021). In recent years, cfDNA has been reported to increase in different physical activities such as light and heavy resistance training (Atamaniuk et al., 2008), sprint interval training (Juškevičiūtė et al., 2023), and moderate intensity cycling (Sawai et al., 2024). It is well-established that high levels of physical activity can lead to metabolic and mechanical muscular damage, leukocyte inflammatory response, and DNA damage due to oxidative stress. These factors, in turn, may result in an increase in circulating cfDNA concentration (Breitbach et al., 2012). Furthermore, the results of our recent study also indicated that eccentric exercise (50 DJs) without an accumulation of metabolites can produce distinct changes in cfDNA levels (Juškevičiūtė et al., 2024). cfDNA is sensitive even to a low impact of metabolic stress (Beiter et al., 2011) but there is no data on how little muscle damage without accumulation of metabolites impact cfDNA levels. Therefore, we aimed to look for a dose-dependent cfDNA response after muscle-damaging exercise (DJs). We examined the time course of plasma cfDNA levels after intermittent 25 and 10 DJs and assessed to what extent cfDNA increases in the absence of metabolic stress. We aimed to test the hypothesis that reducing the number of drop jumps will lead to lower increases in cfDNA levels.

METHODS

This work is a follow-up to the recently published study (Juškevičiūtė et al., 2024), which investigated changes in cfDNA levels in response to primary and secondary muscle damage when muscle damaging exercise was performed without metabolic stress. Most information related to a protocol, physiological measurements and data analysis is similar to that described in the previous article.

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 who did not participate in any official exercise or sports program but were physically active by the standard of participating in recreational physical activity 2–3 times per week. Participants were asked to abstain from any strength and conditioning program 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, 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. As a precaution, participants performed only 1–2 DJs for learning purposes before the trial to avoid muscle damage.

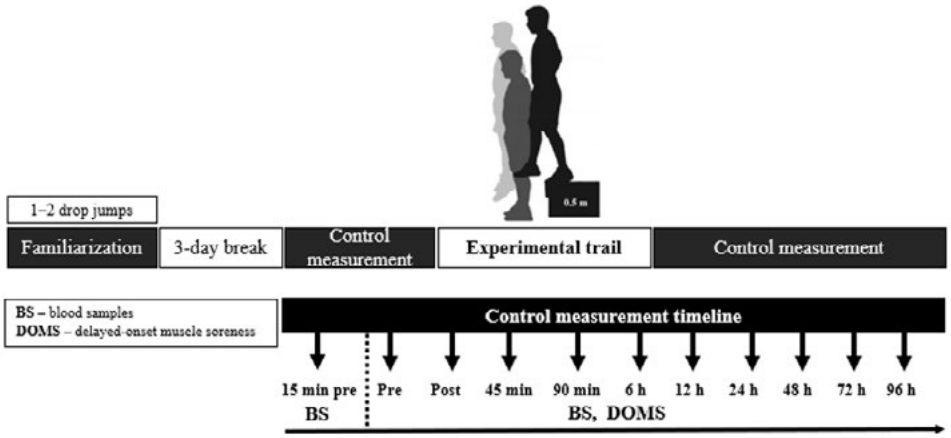
2.3 DROP JUMPS

Eleven men were randomly placed into either a group performing 25 DJs (DJ-25) (mean ± SD; age, 21 ± 1.5 years; weight, 81.1 ± 9.91 kg; height, 186.0 ± 4.96 cm; n = 7) or a group performing 10 DJs (DJ-10) (mean ± SD; age, 22 ± 1.8 years; weight, 79.2 ± 11.0 kg; height, 184.0 ± 8.35 cm; n = 4). Drop jumps were performed at intervals of one every 20 s. The 20 s interval was chosen to minimize metabolic stress.

Each subject underwent exercise testing between 8 and 10 in the morning. Upon arrival at the laboratory, subjects were asked to sit calmly for 10 minutes in the room. Blood samples were taken and then subjects performed an 8–10 min warm-up on an electrically braked cycle ergometer (Ergo-Fit, Pirmasens, Germany) with a pedaling frequency that ranged between 60 and 70 rpm. After warming up, blood samples were collected again (pre-exercise). Following that, the DJs were conducted (they were performed only once on the first day of the study). Blood collections were repeated immediately after exercise and +45 min, +90 min, +6 h and +12 h post-exercise and the following days (24 h, 48 h, 72

h and 96 h post-exercise) (Figure 1). Subjects performed DJs from a 0.5 m height to a 90° knee angle with the immediate maximal vertical rebound 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. A contact mat (Newtest Powertimer Testing system, Oulu, Finland) was used for performing the DJs. For the next four days, blood samples were taken as described below.

Figure 1. **Experimental design. Abbreviations: BS, blood samples; DOMS, delayed-onset muscle soreness.**



Note. Adapted from "Cell-free DNA kinetics in response to muscle-damaging exercise: A drop jump study", Juškevičiūtė et al., 2024 Experimental Physiology, https://doi.org/10.1113/EP091986.

2.5 MUSCLE SORENESS

Participants subjectively assessed muscle soreness (DOMS) using a visual scale of 0 to 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 2–3 squats (Skurvydas et al., 2011). Muscle soreness was assessed pre- and post-exercise and +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 right before exercise, immediately post-exercise and +45 min, +90 min, +6 h, +12 h, +24 h, +48 h, +72 h, +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 16000 × *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 45 min post-exercise using an *Accutrend*® portable lactate analyzer (Roche, Germany). CK activity was measured using an automatic biochemical analyzer (Spotchem EZ SP-4430, Arkray Inc, Kyoto, Japan) at the 11 time points described above.

2.8 QUANTIFICATION OF CFDNA

Concentrations of venous cfDNA were quantified by analyzing unpurified plasma via quantitative real-time polymerase chain reaction (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′-TGCCGCAATAAACATAC-GTG-3′ and 5′-GACCCAGCCATCCCATTAC-3′)

of human long interspersed nuclear elements of the L1PA2 family. Samples were analyzed 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 a SD of the quantification cycle (Cq) >0.4, native plasma samples were rediluted and reanalyzed.

2.9 DATA ANALYSIS

The qPCR data were captured using the CFX Manager Software, version 3.1 (Bio-Rad, Hercules, USA) and Microsoft® Excel, 2016. Statistical analysis was performed using R software, version 4.0.3. Data are presented as the mean ± SD. The assumption of normality was assessed using the Shapiro–Wilk normality test. A significant test was followed by Dunnett's post hoc test or Dunn's test, for normal and non-normally distributed data, respectively. The Dunnettx and Bonferroni adjustment method was used for *p*-value correction, for normally and non-normally distributed data, respectively. The ggplot2 package version 3.2.2 was used for graphical illustrations. We considered p values <0.05 to be statistically significant.

RESULTS

Participants accomplished either 25 or 10 DJs. Blood samples were collected, and subjective reports of muscle soreness were asked before, immediately after, and for the following four days.

3.1. CK ACTIVITY AND DOMS

The average values of plasma creatine kinase before eccentric exercise were similar in both groups (2.01 ± 0.29 (DJ-25) and 2.23 ± 0.45 (DJ-10) IU/L on a logarithmic scale). Plasma CK activity increased after 6, 12, 24 and 48 h post-exercise in the DJ-25 group only ($p = 0.003$, $p < 0.001$, $p < 0.001$, $p = 0.003$, accordingly) (Figure 2).

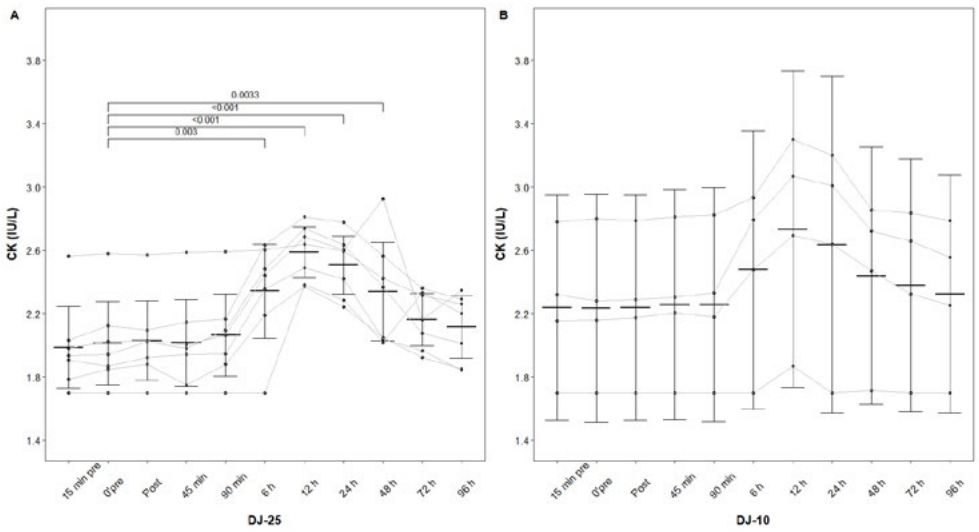


Figure 2. **Creatine kinase over the assessment period (A – DJ-25, B – DJ-10). Data are shown as mean ± SD and individual values a logarithmic scale, n = 7 (DJ-25) and n = 4 (DJ-10). P: differences from the Pre time point. Abbreviations: CK, creatinse kinase.**

At time points 12, 24, and 48 h post-exercise, all subjects reported having DOMS symptoms in the DJ-25 group (4.14 ± 2.48 at 12 h post-exercise ($p < 0.05$), 4.71 ± 2.69 at 24 h post-exercise ($p < 0.01$), 3.86 ± 2.52 at 48 h post-exercise ($p < 0.05$)). No significant increases were noticed in DOMS after 10 DJs over the assessment period.

3.2. LACTATE

We found no increase in lactate concentration immediately post-exercise in both groups. Post-exercise lactate levels were lower compared to pre-exercise levels (from 1.8 ± 0.71 mmol/L (pre-exercise) to 1.27 ± 0.37 mmol/L (post-exercise)) after 25 DJs. The decrease was still evident 45 min post-exercise (1.37 ± 0.37 mmol/L). There was no increase in lactate levels after 10 DJs as well (pre-exercise: 2.2 ± 0.54 mmol/L, post-exercise: 1.97 ± 0.54 mmol/L, 45 min post-exercise: 1.67 ± 0.32 mmol/L).

3.3. PLASMA CFDNA CONCENTRATION

There was a significant increase in cfDNA values immediately post-exercise in the DJ-25 ($p = 0.012$) (Figure 3). As shown in Figure 3, cfDNA concentrations increased noticeably after 12 hours post-exercise but did not significantly differ from the pre-exercise values ($p > 0.05$). No significant changes in cfDNA kinetics were found after 10 DJs ($p > 0.05$).

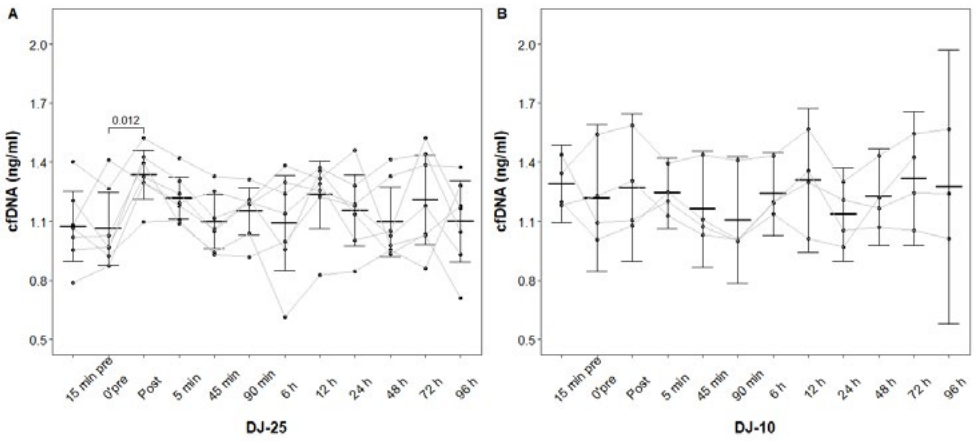


Figure 3. **Cell-free DNA kinetics over the assessment period (A – DJ-25, B – DJ-10). Data are shown as means ± SD on a logarithmic scale, n = 7 (DJ-25) and n = 4 (DJ-10). P: differences from the Pre time point. Abbreviation: cfDNA, cell-free DNA.**

DISCUSSION

The present study explored the effects of 25 and 10 DJs on cfDNA, CK, and DOMS. The findings indicated that cfDNA concentration significantly increased post-exercise only after 25 DJs. CK levels and DOMS were observed to be increased in the 25 DJ group. The absence of lactate accumulation post-exercise suggests that these DJs protocols did not induce metabolic stress.

Regarding cfDNA origins, baseline and post-exercise levels are predominantly derived from hematopoietic-derived cells, as reported in previous research (Neuberger et al., 2022; Moss et al., 2018). Mechanisms such as apoptosis, necrosis, and active secretion contribute to elevated plasma cfDNA concentrations (Breitbach et al., 2012). In the present study, we found a significant increase in post-exercise cfDNA levels after 25 DJs only. While the primary muscle damage caused by 25 DJs was sufficient to affect cfDNA levels in the bloodstream, the level of cfDNA was not altered by 10 DJs. In addition, we did not detect a significant spike in cfDNA levels following secondary muscle damage. Previous work has shown that a higher volume of DJs (e.g., 50 DJs) leads to increased cfDNA following severe primary and secondary muscle damage, suggesting that the extent of damage is crucial for affecting cfDNA levels (Juškevičiūtė et al., 2024). Our findings show that the increase in cfDNA levels is dependent upon the dose of muscle-damaging exercise that is performed.

A significant rise in CK activity was observed in the 25 DJ group at 6-, 12-, 24- and 48-hours post-exercise, with levels not returning to baseline after 96 hours. Notably, there was considerable inter-individual variability in CK responses, especially in the DJ-10 group. Similarly, one study reported wide variations in CK activity ranges (200-2500 IU/L) (Kim & Lee, 2015). The observed increase in CK in the DJ-10 group, though not significant, could result from microtears in myofibrils and the associated inflammatory response trigger muscle nociceptors, leading to DOMS (Baird et al., 2012; Hyldahl & Hubal, 2014). DOMS, associated with inflammatory responses and muscle damage (Peake, Neubauer, et al., 2017), developed following the 25 DJ exercise. Interestingly, at 48 hours post-exercise, the DOMS values were higher after 25 DJs in this study compared to those after 50 DJs in the study by Juškevičiūtė et al. (2024). This observation demonstrates that DOMS exhibits high sensitivity but low specificity.

CONCLUSION

We found that only 25 DJs induced cfDNA concentrations to increase immediately post-exercise, and that a lower volume of DJs (10) is insufficient to cause changes in cfDNA concentrations. The variability in CK responses highlights the complexity of using CK as a sole marker for muscle

damage. Further research with larger sample sizes and varying exercise intensities is recommended to elucidate the relationships between these biomarkers and exercise-induced muscle damage.

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