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The impact of physical training on neutrophil extracellular traps in young male athletes – a pilot study

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ABSTRACT: Neutrophils are an important component of the innate immune response against various pathogens. However, there is a lack of research concerning the effects of short intensive training on neutrophil functions, especially neutrophil extracellular traps (NET) formation. The study aim was to determine the effects of a 19-day training cycle on innate immunity among young male athletes. Six male ice hockey players (< 20 years old) from the Polish national team were monitored across a five-day training camp and after a return to normal club training. The first blood collection took place before training (T1), the second after the training camp (T2) and the third 14 days later (T3). The counts/concentrations of blood biochemical, immune and endocrine markers were compared across each training period. Creatine kinase activity tended to increase at T2 (546 ± 216 $U \cdot L^{-1}$) when compared to T1 (191 ± 111 $U \cdot L^{-1}$; $p=0.063$). Neutrophil extracellular traps formation and neutrophil counts also differed between training periods ($p=0.042$ and $p=0.042$, respectively). Neutrophil counts tended to decrease, in contrast to NET formation which tended to rise, at T2 in comparison to T1 (2.51 ± 0.45 vs 3.04 ± 0.47 $10^9 \cdot L^{-1}$; 24 ± 13 vs 8 ± 15%, respectively). No significant differences in other leucocyte counts were observed. A short period of intensive training was accompanied by some muscle damage and inflammation, as evidenced by CK and NET up-regulation, whilst neutrophil counts were diminished in the blood. Thus, neutrophils and NET could be involved in muscle damage and local inflammatory processes following intensive physical training in young male athletes.

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INTRODUCTION

Neutrophils are an important component of the innate immune response against various pathogens. They are one of the first and most abundant cell populations to reach the affected site after pathogen infections or other inflammatory stimuli [1]. A newly-discovered killing mechanism used by neutrophils is created by an extracellular trap (NET), which is composed of thin chromatin fibers with neutrophil proteins and proteases, such as neutrophil elastase, defensins, cathelicidin (LL-37), myeloperoxidase, cathepsin G and histones [2]. NETs have dual properties in the innate response: 1) acting as an antibacterial agent and 2) involvement in the inflammatory response [1]. Homeostatic interactions between NET formation and removal are relevant for sustaining a healthy immune system [3]. A variety of stimuli are capable of inducing NETs – LPS, IL-1β, IL-6, IL-8, TNF-α, MIF (macrophage migration inhibitory factor), ROS (reactive oxygen species), bacteria, viruses or protozoa [1].

Apart from pathogens, some studies suggest that intensive exercise can also release NETs [4,5]. Syu et al. [5] demonstrated an increase of NET formation after acute exercise on a cycle ergometer until exhaustion in sedentary, but not in active subjects. Beiter et al. [4], however, observed NETs release in endurance-trained and healthy sedentary individuals after 60 min of high-intensity cycling exercise on a cycle ergometer. Others argue that the exercise-induced release of NETs, without the presence of infection or muscle damage, might be a crucial mechanism to maintain immune homeostasis and protect from chronic inflammation [6]. The aberrant release (or ineffective removal) of NETs, as well as disturbance of neutrophil functions

or counts following stressful intensive training, particularly when combined with insufficient recovery, may contribute to increased susceptibility to upper respiratory tract infections, chronic inflammation, excessive muscle/tissue damage or chronic fatigue in athletes [6].

Physical training may also promote exercise-induced muscle damage (EIMD), which leads to an inflammatory response associated with activation of leukocytes, muscle edema, deterioration of muscle function, delayed-onset of muscle soreness (DOMS), and influx of muscle proteins into the circulation [7, 8, 9]. Following induction of muscle damage, neutrophils are rapidly mobilized into the circulation and then migrate and infiltrate into the damaged site, where they remove cellular debris by phagocytosis, ROS, proteolytic enzymes and proinflammatory cytokines [8]. To date, there is a lack of research concerning the effects of short periods of intensive training on neutrophil functions, especially NET formation, where the effects of multiple workouts are likely to accumulative over time.

Therefore, the study aim was to determine the effects of a 19-day training cycle (with different training intensities) on innate immunity among male ice hockey players. Ice hockey is a fast-paced contact sport characterized by intermittent, high-intensity bouts of skating with considerable changes in speed and direction [10]. Such patterns of acceleration and deceleration, coupled with large body impacts, are strong contributors to muscle damage [11]. We hypothesized that training would induce muscle damage and promote an innate immunity response in an intensity-dependent manner, with a return to homeostasis with sufficient recovery.

MATERIALS AND METHODS

Subjects

Six young male ice hockey players (age 18.4 ± 0.6 years, height 1.80 ± 0.05m, body mass 78.7 ± 8.4 kg) were recruited. The participants were all representatives of the Polish National team with each reporting more than 5 years of training experience and thus, were classified as highly-trained athletes. The inclusion criteria were a healthy state (i.e., no injuries, able to perform high-intensity training) at study inception and participation in all blood collections. The exclusion criteria were infection or injury, regular use of medicines (e.g. antiallergic), leaving any blood collection session or iron deficiency. The study was approved by a local Ethics Committee, and written informed consent was obtained from athletes or their parents (KEBN-18-37-JO).

Training

To elucidate whether exposure to intensive training could affect innate immunity, we analysed WBC counts, inflammatory markers and neutrophil extracellular traps in male ice hockey players during a 19-day training camp. The training period consisted of two phases that differed in load intensity: 1) national team training camp (days 1–5) – a phase characterized by high-training loads focusing on development of physical fitness; and 2) friendly matches and club-team training (days 6–19) – a phase characterized by reduced training loads. Training load (in arbitrary units) was estimated by multiplying session intensity, anchored from 0-10 on the CR-10 Rating of Perceived Exertion (RPE) scale, and training session duration [12].

Blood markers

The first blood collection took place at the beginning of the training camp (before start of training sessions – T1), the second at the end of the training camp (T2) and the third collection after the friendly matches and training at home in athletes' clubs (T3). Blood was taken in the morning (between 7 and 9 am) after overnight fasting and a minimum of 12 hours after the last training session. Blood samples were collected from the antecubital vein in a seated position. Haematological and immune assays were performed on the same day, except MPO-DNA complexes, which was determined from frozen blood. Haematological parameters were measured in whole blood collected into EDTA tubes. Blood samples for biochemical measurements were collected into tubes containing coagulation accelerator and serum separator. To separate the serum component for testing, blood samples were centrifuged for 10 min at a speed of 2000 × g. The assays were conducted in the laboratory of the Department of Biochemistry with the implemented quality system (Polish Centre Accreditation no. AB 946).

The mean values of the following biomarkers were measured using a haematology analyser (XN 1000, Sysmex, Japan): platelet volume (MPV), corpuscular volume (MCV), red blood cell distribution

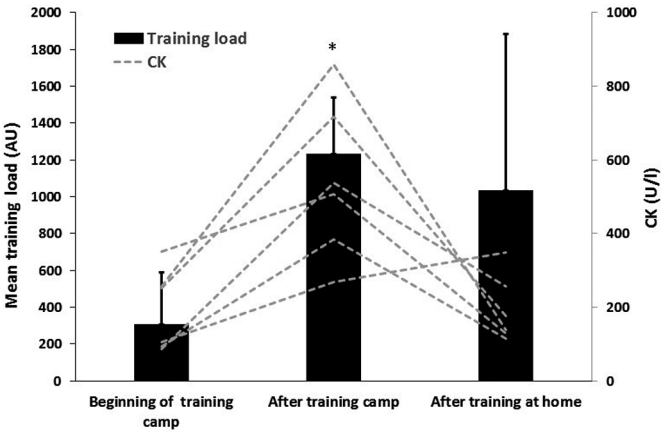

FIG 1. Mean training load (and SD) and individual creatine kinase activity on the beginning and after training camp, as well as, after training at home.
*significantly different from mean training load on the beginning of training camp
T1 – Beginning of training camp
T2 – After training camp
T3 – After training at home

TABLE 1. Descriptive means for blood markers analysed at each time point (n=6).

Markers	Time 1	Time 2	Time 3	Difference between time points p-value		
	Mean ± SD	Mean ± SD	Mean ± SD	1 vs. 2	1 vs. 3	2 vs. 3
Creatine kinase ($U \cdot L^{-1}$)	191 ± 111	546 ± 216	194 ± 91	0.063	1.000	0.130
Uric Acid ($\mu mol \cdot L^{-1}$)	355 ± 69	413 ± 58	329 ± 61	0.745	0.745	0.063
White blood cell ($10^9 \cdot L^{-1}$)	6.93 ± 1.00	6.23 ± 0.84	5.95 ± 0.66	0.745	0.447	1.000
Red blood cell distribution width, as a CV (%)	12.34 ± 0.58	12.63 ± 0.67	12.42 ± 0.59	**0.018**	1.000	0.130
Red blood cell distribution width as a SD (fl)	38.42 ± 2.55	39.98 ± 3.08	39.17 ± 2.90	**0.007**	0.250	0.582
Reticulocyte count ($10^3 \cdot \mu L^{-1}$)	74.33 ± 16.66	63.83 ± 12.97	77.17 ± 12.54	0.063	1.000	**0.012**
Mean corpuscular volume (fl)	85.75 ± 2.37	86.57 ± 2.38	86.47 ± 2.50	0.182	0.337	1.000
Platelets ($10^3 \cdot \mu L^{-1}$)	247.00 ± 54.58	238.67 ± 41.54	239.83 ± 45.84	1.000	1.000	1.000
C-reactive protein ($ng \cdot dl^{-1}$)	0.40 ± 0.28	0.53 ± 0.24	0.20 ± 0.09	0.447	0.447	**0.012**
Mean platelet volume (fl)	11.08 ± 0.55	11.25 ± 0.57	11.40 ± 0.64	0.937	0.130	0.937
Neutrophil count ($10^9 \cdot L^{-1}$)	3.04 ± 0.47	2.51 ± 0.45	2.39 ± 0.40	0.063	0.130	1.000
Lymphocyte count ($10^9 \cdot L^{-1}$)	2.90 ± 0.56	2.86 ± 0.51	2.74 ± 0.43	1.000	1.000	1.000
Monocyte count ($10^9 \cdot L^{-1}$)	0.57 ± 0.11	0.54 ± 0.15	0.50 ± 0.12	1.000	0.182	0.337
Eosinophil count ($10^9 \cdot L^{-1}$)	0.30 ± 0.18	0.26 ± 0.15	0.25 ± 0.14	0.337	0.182	1.000
Basophil count ($10^9 \cdot L^{-1}$)	0.05 ± 0.02	0.05 ± 0.02	0.04 ± 0.01	1.000	0.937	0.337
Neutophil to lymphocyte ratio ($10^9 \cdot L^{-1}$)	1.07 ± 0.21	0.90 ± 0.24	0.88 ± 0.19	0.130	0.447	1.000
Blood cortisol ($nmol \cdot L^{-1}$)	780 ± 176	750 ± 234	505 ± 90	1.000	0.063	0.130

Note: Significant outcomes are highlighted in bold font $p < 0.05$.

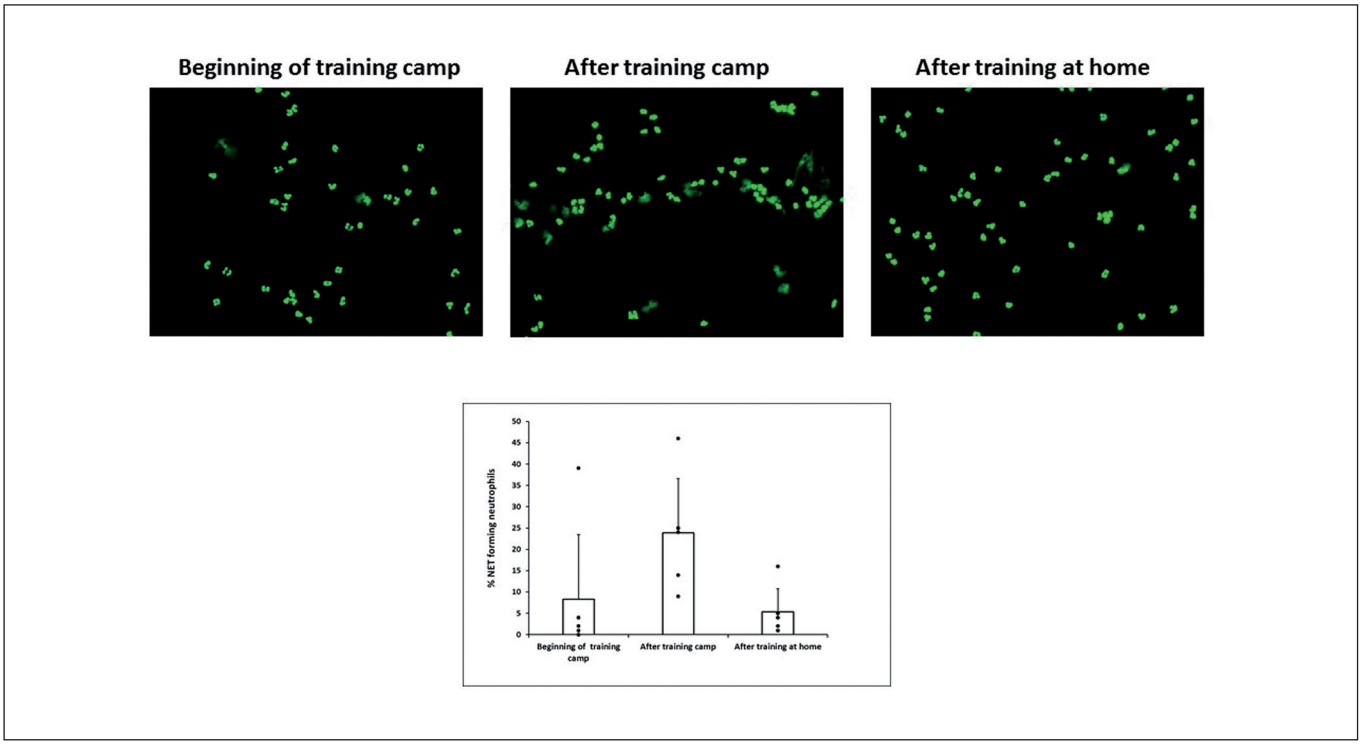

FIG. 2. The bar graph shows quantitation of NET forming neutrophils (and SD) on the beginning and after training camp, as well as, after training at home (p=0.042). Abbreviations: NET – neutrophil extracellular trap; dots – individual percentage of NET forming neutrophils T1 – Beginning of training camp; T2 – After training camp; T3 – After training at home. Representative fluorescence images of NETs in neutrophils from ice hockey players before and after intensive training camp, as well as, after training at home. Neutrophil extracellular traps were fixed and stained with Sytox Green. Magnification = 20X. Abbreviations: NET, neutrophil extracellular trap; T1 – Beginning of training camp; T2 – After training camp; T3 – After training at home.

width as a standard deviation (RDW-SD), red blood cell distribution width as a coefficient of variation (RDW-CV), as well as platelets (PLT), reticulocyte, white blood cell (WBC), neutrophil, lymphocyte, monocyte, eosinophil and basophil counts. C-reactive protein (CRP), creatine kinase (CK) and uric acid (UA) were quantified using the immunoturbidimetric method (for CRP) or the colorimetric method (for CK and UA) with a biochemical analyser (Cobas Integra 400, Roche, Switzerland). All tests were performed with reagent kits from the manufacturer. Serum cortisol concentration was determined using an enzyme-linked immunoassay (ELISA) kit (DRG, Germany).

The microscopy method was employed for NET quantification, because a microplate reader detected both extracellular NET forming cells and intracellular DNA from apoptotic/membrane disrupted cells [5]. For the NET measures, neutrophils were isolated from human whole blood according to the manufacturer's instruction (STEM CELL, Germany). Isolated neutrophils were cytocentrifuged on glass slides and fixed with paraformaldehyde, and NETs stained using Sytox Green. The percentage of NET formation was determined manually by dividing the number of NET-forming neutrophils by the total number of cells in 10 microscopic fields (5 where the number of NETs was the highest and 5 where the number of NETs was the lower) and multiplying the values by 100 [13; 14]. MPO–DNA complexes in serum were measured using an ELISA method, as described [15]. Anti-MPO monoclonal antibody (5μg ml^{-1}; BIO-RAD, cat-no 0400–0002) was coated to 96-well microtiter plates (75 μl per well) overnight at 4°C. After blocking in 1% BSA, serum (40 μl) with the peroxidase-labeled anti-DNA monoclonal antibody (component No. 2 of the Cell Death Detection ELISA Plus. Roche, Cat. No. 11774425001) was added to each well. The samples were shaken at 320 rpm for 2 hours at room temperature, before washing with phosphate-buffered saline, after which the peroxidase substrate was added. After 40 minutes incubation at 37°C in the dark, absorbance was read at a 405-nm wavelength.

Statistical analysis

The non-parametric equivalent of a one-way analysis of variance (i.e. Friedman Test) was used to examine the impact of training intensity (T1, T2, T3) on the immune, biochemical and endocrine markers. Post-hoc comparisons were conducted, where appropriate. Spearman's correlation coefficient was used to test associations between cortisol and neutrophil variables. The level of statistical significance was set at $p < 0.05$. All parameters are expressed as mean ± SD.

RESULTS

As expected, mean training loads were found to be higher at T2 than in T1 (P=0.042) (Figure 1). CK activity also differed between training periods (p=0.042), and trended towards a rising CK level in T2 compared to T1 (p=0.063). An increase in RDW-CV values were observed at T2 versus T1 (p=0.018). RDW SD volume and CRP concentration were elevated at T2, as compared to T1 (p=0.007) or T3 (p=0.012), respectively (Table 1). Reticulocyte count was lower at T2 when compared with T3 (p=0.012) (Table 1).

The percentage of NET forming neutrophils, as well as neutrophil counts, differed between training periods (p=0.042 for both) with a tendency towards a higher amount of NETs after the training camp (T2) in comparison with T1 (p=0.063) (Table 1, Figure 2). Neutrophil counts tended to be lower in T2 from T1 (p=0.063) (Table 1). There were no significant differences in the level of MPO-DNA complexes over time (T1 – 0.390 ± 0.095 O.D.; T2 – 0.406 ± 0.170 O.D.; T3 – 0.351 ± 0.205 O.D.).

DISCUSSION

To the best of our knowledge, no studies have demonstrated an association between the amount of NETs and differences in training intensity among young male athletes. After a short period of intensive exercise (after 5-days of the training camp), where evidence of muscle damage and inflammation emerged (CK up regulation), blood neutrophil counts were found to be diminished and NET formation increased.

Creatine kinase is a widely used biomarker in sport, as an indicator of muscle damage and training load [16, 17]. CK levels peak approximately 24 hours after damaging exercise, but it may remain elevated for up to 7 days or longer, as an indicator of insufficient recovery [17]. In our cohort, CK elevation after the training camp may be a consequence of high training loads and shorter recovery between exercise bouts. If CK exceeds 1000 U·L^{-1}, the inflammatory response is more intense with leukocyte infiltration into injured muscle and recovery usually occurs within a week. Conversely, when CK activity < 1000 U·L^{-1}, inflammation is limited and recovery is usually faster (12–48 h) [8]. Despite CK levels being < 1000 U·L^{-1} in our study, a reduction of neutrophil counts may suggest their inflow to damaged muscle tissues and induction of local inflammatory response. The CK profile of these athletes rose in parallel with greater physical loading (i.e. training camp) before decreasing with reduced loads (i.e. club training). Therefore, it seems reasonable to bind immune system changes with the exercise stimulus and associated muscle damage. Verbal feedback from the team also indicated that, during friendly matches after the training camp, athlete's performance was impaired, as a secondary indicator of muscle damage.

In our study, minor changes in some inflammatory markers [18], such as CRP and RDW were observed. However, these changes were generally small to trivial, such that they are not indicative of illness or acute inflammation. However, they may indicate that intensive training may cause disturbance in the functioning of the immune system.

Accumulation of inflammatory cells in the muscle tissue is the main sign of EIMD [9]. In the present study, only the neutrophil counts tended to decrease after 5 days of intensive training loads, which suggested that neutrophil cells were most affected by intensive exercise among white blood cells. In contrast, Ueno *et al.* [16] did not observe any change in neutrophil counts before and after 5 days of an intensified training camp, and two weeks after the training camp. On the other hand, in healthy untrained adults, neutrophil counts fell below pre-exercise values after 2 and 3 days of acute exercise bout, before gradually returning to basal levels [19].

Neutrophil infiltration and accumulation in muscle can be observed up to 5 days after exercise [20]. The fall in neutrophils may represent part of the inflammatory response to muscle damage caused by intensive exercise. During inflammatory reaction, neutrophils binding to the endothelium prior to their extravasation through capillary walls in the affected tissues. Increased granulocyte adherence and margination are endpoints of acute inflammatory illnesses. The result is a temporary shortening of the intravascular half-life of neutrophils and, if not, occur simultaneous within an increase in their release from bone marrow, possibly causing a transient fall in circulation WBC numbers. In more chronic inflammation, the half-life is prolonged and release of neutrophils (from bone marrow) increases alongside circulating WBC counts [19]. The fall in neutrophils in our study, may be a result of increased migration and accumulation of these cells in muscle-damaged tissue caused by intensive exercise [19, 20].

To our knowledge, the impact of intense multiple physical exercise on NET production has not been studied. Previous research has focused on phagocytic function or the ability to produce reactive oxygen species [16, 21]. However, in the context of NET, only the effect of acute exercise has been studied in humans [4, 5] and mice [22]. In these studies, an increase in NET formation was observed after acute exercise [4, 5]. We observed a disturbance in NET production with training intensity ($p=0.042$). After the training camp, there was an insignificant increase in NET formation versus T1, but no differences were seen at T3. These results partly support the hypothesis that some time is needed for neutrophils to adapt to training loads [23]. MPO-DNA complex levels can also be a confirmation of NET production stimulation after intensive training [22, 24]. Despite the lack of statistical significance, the most MPO-DNA complex levels were observed in T2.

Neutrophil functions can cause damage to healing muscle or delay its regenerative capabilities. For example, exaggerated NET formation could potentially lead to tissue damage during infections because proteins expressed on NETs have antimicrobial, cytotoxic properties and are able to both inactive virus and damage host cells [1]. Therefore, NET formation must be tightly regulated to avoid pathogenesis induced by NET. It was shown that blocking NET formation may result in less tissue damage [25]. In some situations, aggregated NET promotes the ending of neutrophilic inflammation by degrading cytokines and chemokines and disrupting neutrophil recruitment and activation [26]. Some studies suggest that neutrophil-mediated damage may be important, or perhaps necessary, for muscle growth and repair [27]. Nevertheless, it is still unclear how the inflammatory response during muscle tissue injury and repair are determined; therefore, it requires further research [28].

Some suggest that exercise-induced lactic acid accumulation in a physiological state differs from a pathological state [22]. Moreover, the exercise-induced neutrophil phenotype did not cover with the increasing neutrophil subsets during acute inflammation [28]. It is possible that exercise-induced NET accumulation also differs from that seen in a diseased state. This accumulation may be shorter and not so harmful to the body. However, if NETs play a beneficial role in physical exercise, as well as, the expression or etiology of EIMD needs to be studied in more detail.

Ueno *et al.* [16] suggested that a 2-week tapering period seems to be enough time for muscle adaptation and recovery to occur after athletes attended an intensive training camp, which is consistent with the results of our research. At T3 (after training at home), we saw changes that probably reflect the "repeated bout effect", such that skeletal muscle tissue adapts after the first session of damaging exercise. Therefore, repetition of intensive exercise results in less EIMD (less inflammation, oxidative stress, leukocyte infiltration and strength loss) with further exercise exposure [9].

Leukocyte and skeletal muscle interactions must be controlled to avoid prolonged inflammation, excessive tissue damage and fibrosis [9]. Insufficient recovery from EIMD may impair performance. Therefore, monitoring selected indices of muscle status may help athletes and coaches tailor their training/competition strategies and recovery regimens to optimize performance [17]. The response of blood biomarkers in individual athletes may also potentially help coaches understand why some athletes respond differently to the same training stimulus and how to assess individual recovery patterns in the most effective way [29].

In terms of limitations, the small sample size ($n=6$) is problematic, although this was the maximum number of healthy ice hockey athletes we could recruit. Moreover, loss of strength is considered the best functional indicator of EIMD [9]. We did not measure strength directly, but the team reported an impairment in athlete performance during the friendly matches, which aligns to the CK results. As a further limitation, we examined only one function of neutrophil, so we cannot say whether disturbances in NET formation was compensated for by the other neutrophil functions to maintain homeostasis [21]. More frequent sampling and quantitative information about training loads are needed.

CONCLUSIONS

In conclusion, it seems, that a short period of intensive training was accompanied by some muscle damage and inflammation, as evidenced by CK and NET up-regulation, whilst neutrophil counts were found to be diminished. Thus, neutrophils and NET would seem to be involved in muscle damage and local inflammatory processes following intensive physical training in young male athletes.

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Conflicts of interest

The authors declare that they have no conflicts of interes.

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