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ASSESSMENT OF SELECTED EXERCISE-INDUCED CD3^{+} CELL SUBSETS AND CELL DEATH PARAMETERS AMONG SOCCER PLAYERS

PROCENA ODABRANIH CD3^{+} ĆELIJSKIH PODSKUPOVA UZROKOVANIH TRENINGOM
I PARAMETARA ODUMIRANJA ĆELIJA MEĐU FUDBALERIMA

Robert Nowak[1,2], Dorota Kostrzewa-Nowak[1,2]

[1]*Department of Biological Bases of Physical Education, Faculty of Physical Education and Health Promotion,*
University of Szczecin, Szczecin, Poland
[2]*Centre for Human Structural and Functional Research, Faculty of Physical Education and Health Promotion,*
University of Szczecin, Szczecin, Poland

Summary

Background: Molecular mechanisms of biological adaptation to training in professional soccer players are unclear. The aim of this study was to assess the impact of progressive physical effort on peripheral T-cells and their molecular response.

Methods: Thirteen soccer players form Pogo Szczecin S.A., a top league soccer club, (median age 21, range 18–31, years old) performed progressive efficiency tests on a mechanical treadmill until exhaustion at the start (period 1) and the end (period 2) of a competition round. Venous blood T-lymphocyte subsets, selected hallmarks of cell death and plasma cytokine levels were determined by flow cytometry three times: pre-exercise, post-exercise, and in recovery.

Results: Although significant changes in T, Tc and Tc-naïve cell percentages were found in both periods, Th-naïve cell percentages were altered only in period 1. Post-exercise IL-10 plasma levels were higher than pre-exercise, while an increase in TNF-α levels was noticed in recovery from both periods. An increase in recovery IL-12p70 levels was observed in the second period. Increases in the percentage of T-cells with disrupted mitochondrial membrane potentials, elevated levels of phosphorylated H2AX histones and increases in early apoptotic T-cells were also observed.

Kratak sadržaj

Uvod: Molekularni mehanizmi biološke adaptacije na trening profesionalnih fudbalera su nejasni. Cilj ove studije bio je da se proceni uticaj progresivnog fizičkog napora na periferne T-ćelije i njihov molekularni odgovor.

Metode: Trinaest fudbalera iz prvoligaša FK Pogon iz Šćećina (srednja starost 21, raspon 18–31 godina starosti) prošlo je kroz progresivne testove efikasnosti na mehaničkoj traci za trčanje do iscrpljenosti na početku jesenjeg dela prvenstva (period 1) i na kraju (period 2). Podskupovi T-limfocita venske krvi, odabrane karakteristike odumiranja ćelija i nivoi plazma citokina su određivani protočnom citometrijom tri puta: pre treninga, posle treninga i u toku oporavka.

Rezultati: Iako su u oba perioda pronađene značajne promene u procentima ćelija T, Tc i Tc-naivnih ćelija, Th-naivni ćelijski procenti su promenjeni samo u periodu 1. Posle vežbanja, nivoi IL-10 u plazmi bili su veći nego pre vežbanja, dok je povećanje u nivoima TNF-α uočeno tokom oporavka u slučaju oba perioda. Povećanje nivoa IL-12p70 u toku oporavka uočeno je u drugom periodu. Takođe je primećeno povećanje procenta T-ćelija sa poremećenim potencijalom mitohondrijalne membrane, rast nivoa fosforilisanih histona H2AKS i povećanje ranih apoptotskih T ćelija.

Address for correspondence:

Robert Nowak
Department of Biological Bases of Physical Education,
Faculty of Physical Education and Health Promotion,
University of Szczecin
40B Piastów Al., building 6
71-065 Szczecin, Poland

List of abbreviations: APC, allophycocyanin; BMI, body mass index; BMR, basal metabolic rate; DSB, DNA double-strand breaks; EDTA, ethylenediaminetetraacetic acid; FAT, percentage of fat; FITC, fluorescein isothiocyanate; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolcarbocyanine iodide; MET, metabolic equivalent; PBMCs, peripheral blood mononuclear cells; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; RC, respiratory compensation; Rf, respiratory frequency; ROS, reactive oxygen species; RQ, respiratory quotient; RT, room temperature; SSBs, DNA single-strand breaks; TBW, total body water; TNF-α, tumour necrosis factor alfa; V_E, maximum ventilation; VO_2max, maximum oxygen uptake.

Conclusions: The immune system in soccer players creates space for naïve CD3$^+$CD8$^+$ cells by inducing mechanisms of cell death. It seems that the cumulative effect of physical activity during a competition round induced an adaptive mechanism, since the cell death process was induced faster during period 2.

Keywords: apoptosis, flow cytometry, progressive effort, soccer, T-lymphocytes

Zaključak: Imuni sistem kod fudbalera stvara prostor za naivne CD3$^+$CD8$^+$ ćelije indukovanjem mehanizama ćelijske smrti. Čini se da je kumulativni efekat fizičke aktivnosti za vreme takmičenja izazvao stvaranje adaptivnog mehanizma, jer je proces ćelijske smrti indukovan brže tokom perioda 2.

Ključne reči: apoptoza, protočna citometrija, progresivni napor, fudbal, T-limfociti

Introduction

Soccer is one of the most popular team sports in Europe. The physical effort in soccer is found to be of mixed type (aerobic and anaerobic) which is associated with different player activities during the match. The high number of accelerations and decelerations, associated with high dynamics of play, create an exceptional burden for muscles (1, 2). Due to the different types of exercise, players must adapt to generate energy from both anaerobic and aerobic metabolism (3). Moreover, physical effort is the main cause of disorders in antioxidant balance related to increases in reactive oxygen species (ROS) generation and damage caused by ROS at the cellular level (4). It is worth noting that there is a close connection between muscles/blood circulation regarding alterations in immunological variables, especially in peripheral blood mononuclear cells (PBMCs), namely monocytes (5). Another aspect requiring adaptation is the power and strength needed during the game to allow the player to use a variety of their skills (2, 3), which also might affect immunological cell levels.

Previous research has emphasized that long-term training can influence an athletes' cellular metabolism which can lead to muscle damage and induce oxidative stress (4, 6). The influence of physical efforts on an athlete's antioxidant status and modulation of immune cells has been described (7–9). DNA base residue damage leads to DNA single-strand breaks (SSBs). The accumulation of DNA SSB damage, observed in the case of repair system failure, causes genome instability favouring the accumulation of double-strand breaks (DSBs) leading to cell death (10). Literature reports have shown that repeated intense interval-exercise appears to influence induction and execution of cell death, especially apoptosis, in PMBCs. Although there are numerous literature data describing the effect of different types of exercise in usually sedentary subjects, there is still a need to better understand the molecular mechanisms of biological adaptation to the training process in professional athletes. There is data which describes different causes of overtraining syndrome (11, 12), and it is well known that prolonged tiredness in athletes may lead to immunosuppression (8, 12, 13). However, it is not clear whether or not described decreases in leukocytes (especially lymphocytes) during cumulative training loads is related with induction and execution of apoptosis and/or lack of efficient hematopoietic capacity. During the recovery time, peripheral blood mononuclear cells behave differently with regard to cell death and migration (14). Different subsets of lymphocytes respond in different ways with respect to intensity, cell counts, and markers of apoptosis and cell migration. Cells expressing CD4 and CD8 markers appear to be prone to apoptosis after moderate exercise, but significant increases in migration at higher exercise intensities suggest movement of these cells from the vasculature in post-exercise measurements (15). The aim of this study was the assessment of the impact of maximal effort on peripheral T-cell distributions and their molecular response during exhaustive efforts.

Materials and Methods

Study design

The study was designed to better understand the influence of exhaustive effort on T-cells in peripheral blood as a comparison of two preparatory periods during league competition rounds among top league soccer players.

Total lymphocyte percentages and selected T-cell (CD3$^+$) subsets: T-helper cells, CD4$^+$ (Th); T-suppressors/cytotoxic, CD8$^+$ (Tc); T-naïve CD4$^+$ or CD8$^+$ cells, respectively, distributions before, immediately after the progressive tests, and during recovery times (about 17 hours after the test as an average time between two consecutive training units or the soccer match and the following training unit) were determined. Additionally, mitochondrial membrane potentials, H2AX histone phosphorylation related to DNA damage, and induction and execution of apoptosis were determined in analysed T-cells. Cardio-respiratory fitness measures: maximum oxygen uptake (VO$_2$max), maximum ventilation (V$_E$), metabolic equivalent (MET), respiratory quotient (RQ; volume ratio of emitted CO$_2$ to oxygen uptake) and respiratory frequency (Rf) were also determined.

The studies were performed at the beginning and the end of autumn competition rounds among soccer players, at the same time of day ± 1 hour. Laboratory conditions, including the order of participants, were the same for both periods. The studies were performed in the Centre for Human Structural

and Functional Research, Faculty of Physical Education and Health Promotion, University of Szczecin, Poland.

Participants

Thirteen participants (median age 21, range 18–31, years old) were recruited from Pogon Szczecin S.A., a top league soccer club in Poland. All soccer players qualified for the study were playing in a midfielder or defender position through the duration of the experiment and were on the field not less than 22.5 hours (the participants played in matches not less than 80 minutes per match). They had no history of any metabolic syndrome (according to the International Diabetes Federation definition, i.e. without diabetes, prediabetes, abdominal obesity, high cholesterol and/or high blood pressure) or cardiovascular diseases (as defined by WHO as disorders of the heart and blood vessels). Participants were non-smokers and refrained from taking any medications or supplements known to affect metabolism. They were fully informed of any risks and discomfort associated with the experimental procedures before giving their consent to participate. The study was approved by the Local Ethics Committee at the Regional Medical Chamber in Szczecin in accordance with the Helsinki Declaration.

Progressive tests on a mechanical treadmill

Progressive efficiency tests on a mechanical treadmill until exhaustion are routinely applied in sports practice because of the possibility of simultaneously using a stationary breath-by-breath gas-exchange data analyser to determine cardiorespiratory fitness measured as a function of increasing fatigue. The test started with 5 minutes of warm-up running at a speed of 5 km/h. During the main test, the inclination of the treadmill was 1% and the speed increased by 1 km/h after every 2 minutes of the test until exhaustion. The cardiorespiratory fitness measures: VO_2max, V_E, MET, RQ, and Rf were determined using a state-of-the-art breath-by-breath gas-exchange data analyser (Quark CPET; Cosmed, Albano Laziale, Italy) (16).

Blood sampling

Blood samples were obtained three times from the elbow vein: before testing (pre-exercise), no longer than 5 minutes after the test (post-exercise) and about 17 hours after the test which was at the end of recovery time (recovery). At each time, blood samples were taken into 7.5 mL S-Monovette tubes with ethylenediaminetetraacetic acid (EDTA K_3, 1.6 mg EDTA/mL blood) (SARSTEDT AG & Co., Nümbrecht, Germany). All analyses were performed immediately after blood collection. Importantly, for the safety of the participants, the test protocol required them to be carried out after a light breakfast. Therefore, the pre-exercise and post-exercise blood samples were not fasting blood samples. No food was taken before the end of the recovery period.

PBMC isolation

To obtain peripheral blood mononuclear cells, whole blood samples were centrifuged for 15 min at $2500 \times g$, room temperature (RT). The buffy coat was transferred to Lymphocyte Separation Medium (density 1.077–1.080 g/mL at 20 °C) (Corning, Manassas, VA, USA) and centrifuged 40 min at $350 \times g$, RT. The obtained PBMC fraction was washed using Stain buffer (BD Pharmingen™, San Jose, CA, USA) and used for further analyses.

Flow cytometric analyses

All flow cytometry analyses were performed using a BD Accuri™ C6 flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and the results were calculated using BD Accuri™ C6 software (ver. 1.0.264.21) unless otherwise stated.

Blood phenotyping

Whole blood lymphocyte phenotyping was performed using a BD Multitest™ IMK kit (BD Biosciences, San Jose, CA, USA). The expression of surface markers was determined according to the manufacturer's protocol. Briefly, an antibody cocktail was used to determine the percentage of T-lymphocyte subsets in erythrocyte-lysed blood samples. The cocktail contained antibodies including fluorescein isothiocyanate (FITC)-labelled anti-CD3, clone SK7; phycoerythrin (PE)-labelled anti-CD8, clone SK1; peridinin chlorophyll protein (PerCP)-labelled anti-CD45, clone 2D1 (HLe-1) and allophycocyanin (APC)-labelled anti-CD4. For each sample, the fluorescence signal of at least 10^4 total events was measured.

CD3$^+$ naïve T-cell subset phenotyping

The analysis of CD3$^+$ naïve T-cell subsets in isolated PBMCs was performed using a Human Naïve/Memory T Cell Panel (BD Pharmingen™) and anti-CD8$^+$ fluorescent labelled antibodies (BD Pharmingen™) according to the manufacturer's protocol. Briefly, two different cocktails of antibodies (for CD4$^+$ and CD8$^+$ cells, respectively) containing Alexa Fluor® 647-labelled Mouse Anti-Human CD197 (CCR7), PerCP-Cy™ 5.5-labelled Mouse Anti-Human CD4, or PE-labelled anti-CD8 and FITC-labelled Mouse Anti-Human CD45RA, were prepared prior to

use. For each sample, the fluorescence signal of at least 5×10^4 ungated events was measured. The analysed CD4$^+$ SSC^{low} or CD8$^+$ SSC^{low} cells were determined as T-naïve (CD45RA$^+$CD197$^+$).

Selected cytokine profile

Measurements of selected plasma cytokines, namely interleukin-8 (IL-8), interleukin-6 (IL-6), inter-leukin-10 (IL-10), tumour necrosis factor alpha (TNF-α), and interleukin-12p70 (IL-12p70) protein levels were performed using a BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (BD Biosciences) according to the manufacturer's protocol. For each sample, the fluorescence signal of 2100 events, gated for the beads population, was measured. Results were calculated using FCAP Array™ Software (ver. 3.0.1; Soft Flow Hungary Ltd., Pecs, Hungary).

H2AX phosphorylation analysis

Phosphorylation of H2AX (γ-H2AX; pS139) histone in isolated PBMCs was assessed using PE-labelled Mouse Anti-H2AX (pS139) antibodies (BD Pharmingen™). Briefly, about 10^6 cells were fixed using BD Cytofix™ Fixation buffer (BD Biosciences) and washed with BD Pharm Lyse™ Lysing Buffer (BD Pharmingen™). Afterwards, the cells were incubated with PE-labelled Mouse Anti-H2AX (pS139), clone N1-431 antibodies (BD Pharmingen™). Additionally, FITC-labelled anti-CD3, clone SK7 antibodies (BD Biosciences) were used for further identification of the T-lymphocytes. For each sample, the fluorescence signal of at least 10^4 events, gated for the forward and side light-scatter characteristics of lymphocytes, was measured.

Mitochondrial membrane potential analysis

The analysis of mitochondrial membrane potential ($\Delta\Psi_m$) in isolated PBMCs was performed using a BD™ MitoScreen Kit (BD Biosciences) according to the manufacturer's protocol.

Briefly, about 10^6 cells were incubated with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide). Additionally, PerCP-labelled anti-CD3, clone SK7 antibodies (BD Biosciences) were used for further identification of the T-lymphocytes. For each sample, the fluorescence signal of at least 10^4 events, gated for the forward and side light-scatter characteristics of lymphocytes, was measured.

Apoptosis assessments

The degree of apoptosis in analysed isolated PBMCs was assessed using APC-labelled Annexin V (BD Pharmingen™) and propidium iodide (Sigma-

Aldrich, Saint Louis, Missouri, USA). Briefly, about 10^6 cells were incubated with using APC-labelled Annexin V and propidium iodide (final concentration 5 µg/mL). After washing the cells with Stain Buffer (FBS) (BD Pharmingen™). FITC-labelled anti-CD3, clone SK7 antibodies (BD Biosciences) were used for further identification of the T lymphocytes. For each sample, the fluorescence signal of at least 10^4 events, gated for the forward and side light-scatter characteristics of lymphocytes, was measured.

Statistical analyses

All data are presented as medians (interquartile ranges). Statistical analysis was performed using Statistica, version 13 (TIBCO Software Inc., 2017). The significance of differences observed between the beginning and end of the competition round were assessed using Wilcoxon's matched-pairs test. The significance of differences observed between analysed time points (pre-exercise vs. post-exercise vs. recovery) was assessed using Friedman's analysis of variance followed by post-hoc Dunn's tests with Bonferroni correction. Each time, $p < 0.05$ was considered a significant difference.

Results

The cardiorespiratory fitness values found in both periods (at the beginning and the end of the competition round) were similar for studied participants (Table I) providing evidence that there were no changes in their physiological capacity.

Table I Cardiorespiratory fitness measures of soccer players during progressive tests until exhaustion.

	Period 1. The beginning of the competition round	Period 2. The end of the competition round
	n=13	
VO$_2$max (mL/kg/min)	58.7 (56.8–60.7)	58.7 (57.3–60.1)
VE (L/min)	113.0 (105.6–134.9)	120.0 (113.0–131.2)
MET (mL/kg/min)	16.3 (15.8–16.6)	16.0 (15.4–16.6)
RQ	1.14 (1.11–1.18)	1.15 (1.14–1.18)
Rf	58.8 (47.6–63.2)	60.2 (58.8–63.2)

The table presents medians (Q1–Q3) values.
The analyses were performed using a state-of-the-art breath-by-breath gas-exchange data analyser Quark CPET (Cosmed, Albano Laziale, Italy).
n – number of participants, VO$_2$max – maximum oxygen uptake; V$_E$ – minute ventilation; MET – metabolic equivalent; RQ – respiratory quotient (volume ratio of emitted CO$_2$ to oxygen uptake); Rf – respiratory frequency.

Table II T cell subsets and plasma cytokine levels of soccer players during progressive tests until exhaustion.

	Period 1. The beginning of the competition round			Period 2. The end of the competition round		
	$n=13$					
	pre-exercise	post-exercise	recovery	pre-exercise	post-exercise	recovery
Total lymphocytes (%)	23.4 (18.9–28.3)	29.1 (26.9–33.4)	27.0 (20.8–29.0)	23.8 (15.7–34.9) a)**	29.9 (28.2–31.4) b)***	26.5 (22.0–26.9)
T-cells (%)	65.5 (62.3–71.9) a)**	59.1 (53.8–60.7) b)****	68.3 (65.7–74.1)	68.9 (62.1–71.4)	61.3 (55.0–65.3) b)*****	74.6 (67.4–75.7) c)*
Tc-naïve cells (%)	61.2 (46.8–71.5)	46.3 (35.4–58.1) b)****	72.8 (50.3–81.0)	60.5 (54.6–63.1)	53.5 (43.7–56.0) b)*****	68.0 (64.9–75.6) c)**
Th-cells (%)	57.5 (52.1–60.5) a)**	48.4 (45.3–52.1) b)***	56.8 (45.2–64.3)	52.5 (49.3–56.9) a)*	46.2 (43.2–52.7) b)***	59.1 (53.3–61.6)
Th-naïve cells (%)	62.1 (56.8–73.7)	60.0 (52.1–65.8) b)**	73.7 (59.2–74.6)	55.9 (51.7–64.4)	52.3 (42.9–57.7)	50.7 (46.1–60.5)
IL-10 (pg/mL)	0.87 (0.37–1.13) a)**	2.40 (1.99–2.70)	2.13 (1.32–2.36)	1.78 (1.24–2.03) a)**	2.89 (2.38–4.20)	4.05 (3.59–4.58) c)****
TNF-α (pg/mL)	2.46 (1.99–2.79)	2.99 (2.14–3.26)	3.65 (2.79–3.65) c)*	2.47 (1.98–2.78) a)**	4.87 (4.57–5.02)	4.98 (4.57–6.59) c)***
IL-12p70 (pg/mL)	2.32 (1.80–2.45)	1.99 (1.65–2.65)	2.36 (1.70–2.65)	2.18 (1.89–2.58)	3.97 (2.98–4.58)	6.42 (5.64–7.89) c)*****
IL-6 (pg/mL)	1.03 (0.25–2.36)	1.25 (0.72–1.36)	1.26 (0.36–2.15)	1.58 (1.23–1.87)	1.58 (1.25–1.99)	2.03 (1.58–2.16)

The table presents median (interquartile range) values.
Significance levels of differences observed between analysed time points (pre-test vs. post-test vs. recovery) were assessed using Friedman's analysis of variance followed by post-hoc Dunn's tests with Bonferroni correction.
The results of post-hoc analyses: a) pre-exercise vs. post-exercise; b) post-exercise vs. recovery; c) pre-exercise vs. recovery.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; ***** $p<0.00001$
n – number of participants.

Table III Parameters of T-lymphocytes' cell death in soccer players during progressive tests until exhaustion.

	Period 1. The beginning of the competition round			Period 2. The end of the competition round		
	$n = 13$					
	pre-exercise	post-exercise	recovery	pre-exercise	post-exercise	recovery
T-cells with disrupted ΔΨm (%)	0.06 (0.00–0.93) a)**	13.79 (13.04–23.75)	22.57 (16.72–26.77) c)****	3.68 (1.79–6.05) a)**	19.55 (18.41–26.23) b)****	1.34 (0.56–3.38)
T-cells with DNA damage (as fosforylated H2AX histones present) (%)	5.6 (1.4–13.2)	0.1 (0.0–3.9) b)***	21.0 (16.3–32.5)	0.6 (0.3–2.1)	5.4 (2.5–7.3) b)*	17.2 (13.7–18.7) c)*****
Live T-cells (%)	94.5 (90.2–96.1)	94.4 (93.2–95.7) b)***	85.0 (84.2–86.4) c)**	98.7 (98.4–98.9) a)*****	94.5 (93.8–95.4)	96.9 (96.7–97.4) c)**
Early apoptotic T-cells (%)	4.8 (3.5–6.1)	5.1 (3.9–6.5) b)***	14.8 (13.3–15.6) c)**	1.2 (1.0–1.6) a)*	5.1 (4.6–6.0) b)*	9.5 (8.7–10.7) c)*****
Late apoptotic T-cells (%)	0.2 (0.1–0.3)	0.1 (0.1–0.15)	0.1 (0.1–0.14)	0.1 (0.0–0.01)	0.0 (0.0–0.02) b)***	2.1 (1.8–2.4) c)***
Necrotic T-cells (%)	0.2 (0.1–0.2)	0.2 (0.1–0.3)	0.1 (0.1–0.2)	0.1 (0.0–0.1)	0.1 (0.0–0.2)	0.2 (0.1–0.2)

The table presents median (interquartile range) values.
Significance levels of differences observed between analysed time points (pre-test vs. post-test vs. recovery) were assessed using Friedman's analysis of variance followed by post-hoc Dunn's tests with Bonferroni correction.
The results of post-hoc analyses: a) pre-exercixe vs. post-exercise; b) post-exercise vs. recovery; c) pre-exercise vs. recovery.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ***** $p < 0.00001$
n – number of participants.

The exhaustive effort caused changes in percentages of T-cell subsets (*Table II*). Significant increases in T, Tc and Tc-naïve cell percentages in recovery as compared to post-effort values were found in both periods, Th-naïve cell percentages were only altered during period 1.

Post-effort IL-10 plasma levels were higher than baseline values, while significant increases in TNF-α levels were noticed during recovery time in both periods. Significant increases in IL-12p70 recovery levels were noted only at the end of the competition round, and no changes in IL-6 level were observed (*Table II*).

Post-effort decreases in T-cell percentages were accompanied by significant increases in the percentage of T-cells with disrupted $\Delta\Psi_m$. Interestingly, the increase in cells with disrupted $\Delta\Psi_m$ was also observed in the recovery time-point of period 1 (*Table III*). The levels of phosphorylated H2AX histones, related to DNA-damage-repair processes in analysed T-cells, was elevated in recovery times. The decreases in live T-cells observed was a consequence of increases in early apoptotic T-cell percentages. Remarkably, at the beginning of the competition round, this increase was observed in the recovery time, while at the end of the competition round, this was observed after exercise and during recovery. Also, increases in late apoptotic CD3$^+$ cells were observed in the recovery time at the end of the competition round (*Table III*).

Discussion

Cell death provides a universal mechanism of tissue development including immune system cells. Thanks to this phenomenon properly developed cells are selected, getting rid of all other damaged and/or dangerous cells (9, 17). It is commonly accepted that regular physical activities can be related to well-being and cause an anti-inflammation effect both in animal models and humans (7, 18, 19). On the other hand, a cumulative effect of intense physical effort can cause an immunosuppressive effect, especially among elite athletes (8, 20, 21). It was evidenced in our study that the immune response induced by exhaustive effort was more rapid at the beginning than at the end of the competition round.

Decreases in senescent CD4$^+$ and CD8$^+$ T-lymphocyte percentages after maximal exercise has been described in trained athletes compared with an untrained group (22). The post-exercise decreases in Th-cell percentages observed in our study were compensated during recovery at both periods. No changes in Tc-cells both post-exercise and during recovery, in comparison to baseline values, were observed. According to Simpson (23), the immune system in regularly training subjects creates space for naïve T-lymphocytes by mobilisation of senescent T-cells. The results found in our study are in line with this hypothesis. Spielmann et al. (24) observed that

resting athletes with high VO$_2$max (maximal aerobic capacity) had more naïve CD8$^+$ and less senescent CD4$^+$ and CD8$^+$ T-cells as compared to participants with lower VO$_2$max values. Our study showed that increases in naïve Th (CD4$^+$) cells took place in recovery only at the period 1 (the beginning of the competition round), while naïve Tc (CD8$^+$) cells increased in recovery in both periods (compared to post-exercise values). This may suggest that exhaustive effort performed at the end of the competition round did not induce regulatory pathways related to Th-cells. On the other hand, it also might suggest that cumulative effects of intense physical activity during the whole competition round induced an adaptive mechanism in the elite soccer players immune system and did not cause a rapid inflammation effect.

To verify the effect of maximal effort on an organism's inflammation state, plasma levels of selected cytokines were determined. It was evidenced that IL-10 and TNF-α serum levels increased significantly after exhaustive effort or during the recovery time, respectively. The observed changes in TNF-α levels are most likely related to an inflammatory response to muscle damage caused by exhaustive effort (25). This involves IL-10 as an anti-inflammatory response to exercise (26, 27), playing a crucial role in muscle tissue regeneration (28–30). Our results are in line with the suggestion that increased IL-10 plasma concentration after exercise is required for the reduction of muscle damage and tissue recovery (25, 28). The increases in IL-12p70 levels found at the end of the competition round may suggest an important role of Tc-cells in the creation of post-exercise immune response patterns among soccer players. Interestingly, our previous study (using other test protocols, e.g. no inclination and other treadmill acceleration configuration) provided evidence that Th-cells are recruited and involved in post-exercise modulation of the immune system in soccer players (31).

Simpson's hypothesis provides an explanation of the phenomenon of increasing T-naïve cells by a negative feedback loop: decrease in peripheral T-lymphocytes is caused by apoptosis of senescent T-cells, and then this gap is filled by naïve T-lymphocytes leaving the thymus (23). Significant increases in T-cells with disrupted mitochondrial membrane potentials ($\Delta\Psi_m$) after exhaustive effort were observed in our study. Moreover, increases in T-cells with the presence of phosphorylated H2AX histones, indicating DNA damage, were also observed. Interestingly, it was found that the disruption of $\Delta\Psi_m$ and H2AX phosphorylation levels in T-cells during the recovery time was more elevated at the beginning than at the end of the competition round. Unrepaired DNA damage is a factor inducing apoptosis. Our study showed significant increases in early apoptotic T-cells post-exercise and during recovery in both periods, while the increase in late apoptotic T-cells was observed only in period 2. This may suggest that mechanisms of cell

death are induced faster later on, related to biological adaptation to the physical effort during the whole competition round. Krüger et al. (9) also described exercise-induced increases in apoptosis of low differentiated T-cells related to changes of circulating T-cells and progenitor cells among sedentary subjects. Taking Simpson's hypothesis (23) and Brown et al.'s (22) observations that changes in distributions between naïve and senescent T-cells are more intense in regularly training participants (22, 24) into account, the results of our study support this theory.

The results of our study provided evidence that the immune system in soccer players creates space for naïve $CD3^+CD8^+$ cells by inducing mechanisms of cell death in analyzed cells. DNA damage and apoptosis induction in $CD3^+$ cells may explain the fluctuation of post-exercise T-cell subsets' distributions by negative feedback loops as described by Simpson (23).

It seems that the cumulative effects of physical activity during the whole competition round induced an adaptive mechanism in elite soccer players' immune systems and did not cause rapid inflammation effects. Biological adaptation to the physical effort during the whole competition round can be assumed by the fact that mechanisms of cell death were induced faster at the end of the competition round.

Practical implications

In the authors' opinion, this study could broaden the knowledge regarding molecular responses to exhaustive effort and help to understand better »The Open Window Theory«. From the practical point of view, trainers are highly interested in adapting the training process to avoid, e.g. upper respiratory tract infections as a result of high-intensity exercise-related immunosuppression. Any infection within a sports team weakens the whole team. On the other hand, proper training loads are needed to induce the microinflammation anabolic effects of the immune system that could enhance an athlete's performance. Moreover, it seems that maximal effort on a treadmill could provide equivalent training unit, recruiting the immune system to trigger compensation pathways needed for abovementioned anabolic effects. However, it should be emphasized that this hypothesis needs further investigation.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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