



Circulating Inflammatory Biomarkers and Endocrine Responses to Exercise in Female Soccer Players

by

Grażyna Janikowska¹, Aleksandra Kochańska-Dziurowicz², Ilona Pokora³,
Aleksandra Żebrowska³

The objective of the study to determine the effects of graded exercise on the cytokines and insulin-like growth factor-1 (IGF-1), growth hormone (hGH), testosterone (T), and cortisol (C) concentrations in the peripheral blood of female soccer players, and to evaluate if increased inflammatory biomarkers were related to these hormones and performance variables. Sixteen female soccer players (N = 16, age 19.3 ± 2.3 years) participated in this study. Blood samples were collected at three time points: pre-exercise, post-exercise, and in the 15th minute of recovery, to evaluate morphological and biochemical variables. The relative expression of IL-6 (interleukin 6) and serum concentrations of the cytokines were increased in the recovery period compared to pre-exercise levels (p = 0.03 and p=0.005, respectively). There was a significant effect of exercise on serum hGH level (p < 0.001), T/C ratio (p = 0.001), and C level (p=0.02). Positive correlations were found between: post-exercise IL-1β (interleukin 1 beta) and IL-6 (R = 0.84, p = 0.000), and the IL-6 and TNF-α (tumor necrosis factor alpha) gene expression during recovery (R = 0.65, p = 0.009), and serum IL-1β post-exercise and maximal power (R = 0.68; p = 0.004). Exercise-induced serum C levels positively correlated with IGF-1 levels (R = 0.52 p = 0.05). Negative associations were revealed between post-exercise T/C ratio and IGF-1 (R = -0.58, p = 0.03) and serum free T and IL-β (R = -0.56, p = 0.04) levels. The low level of pre-exercise genes and protein of the IL-1β, IL-6 and TNF-α indicate a lack of inflammation signs in the female soccer players. This study shows significant effects of exercise on hormone levels and pro-inflammatory markers, which could be used to identify the role of female sex steroids on the immune function.

Key words: cytokine, hormones, female soccer players, exercise performance.

Introduction

The increasing popularity of female soccer developed an interest regarding their physiology and adaptive responses to exercise. Especially to exercise-induced secretion of pro-inflammatory cytokines and steroid hormones during different training loads and competition (Roli et al., 2018; Edwards et al., 2013; Walsh et al., 2011). There is extensive literature regarding the variation of different biological variables, resulting from physical and mental stress of professional athletes (Ostrowski et al., 1999; Rowel et al., 2018; Oliveira et al., 2009). The accumulated effect of exercise on

systemic inflammation has been studied widely in male soccer players (Bizzini and Dvorak, 2015). However, the knowledge about the relation between blood cytokine gene expression, anabolic and catabolic hormone concentrations and the performance variables in female athletes is still inadequate (Gjevestad et al., 2015; Larsen et al., 2018; Gillum et al., 2011).

Cytokines, including interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), are important mediators of acute phase response to muscle damage and

¹ - Department of Analytical Chemistry, Medical University of Silesia, Katowice, Poland.

² - Silesian College of Medicine in Katowice, Poland.

³ - Institute of Sport Sciences, Academy of Physical Education, Katowice, Poland.

muscle contractions (Ostrowski et al., 1999, Walsh et al., 2011; Scheller et al., 2011). The relationship between the mentioned cytokines is well-known; where IL-6 inhibits the expression of TNF- α and IL-1 β . In response to exercise IL-6 is produced in larger amounts than the other cytokines (Petersen and Pedersen, 2006) and a positive correlation between exercise intensity and plasma IL-6 concentration has also been reported (Ostrowski et al., 2000).

Numerous studies have shown different levels of blood cytokines depending on the type and intensity of exercise and training status (Petersen and Pedersen, 2006; Meckel et al., 2011). Additionally, during exercise, cytokine secretion is related to endocrine and paracrine factors (Pedersen and Febbraio, 2008; Meckel et al., 2011). Given the relationship between hormones and immunity, little research has investigated the role of female sex steroids on the immune function (Schuurs and Verheul, 1990; Giraldo et al., 2011; Roli et al., 2018). A greater anti-inflammatory status has been reported in response to contraceptive use and was parallel with lower estrogens concentrations. Cortisol, prolactin, and IL-6 seem to be involved in gender-inflammatory differences (Gillum et al., 2011), and in the restoration of homeostasis (Jürimäe et al., 2011). It has been hypothesized that, in female athletes exercise may lead to an higher increase of anabolic hormones in response to exercise-induced pro-inflammatory cytokines (IL-6, TNF α) as well as an higher anti-inflammatory cytokine level compared to reference norms in a healthy population (Roli et al., 2018).

High-intensity exercise has been known to increase circulating IGF-1, one of the most important anabolic factors, that takes place following exercise below and above the anabolic threshold (Copeland and Heggie, 2008; Żebrowska et al., 2009; Hackney et al., 2016). Exercise-associated increase in testosterone (T) and T/C ratio, might potentially influence muscle protein synthesis, anti-inflammatory properties, and GH/IGF-1 axis response to exercise (Chmura et al., 2019, Oliveira et al., 2009). It has been hypothesized that testosterone induced a reduction in circulating IGF-1 levels and may have a direct, IGF-1 and GH-independent effect on muscle growth. Importantly, the balance between the anabolic and inflammatory response

to exercise has been suggested as a good predictor of the effectiveness of exercise on health benefits (Gregory et al., 2013; Jürimäe et al., 2011; Smith, 2000).

The peripheral inflammatory response to intense exercise has been extensively studied in male soccer players. In contrast, relatively little is known concerning the hormonal changes that occur following high intensity exercise and its implication on the potential anti-inflammatory effects in female athletes (Soligard et al., 2008; Andersson et al., 2010; Souglis et al., 2015).

Therefore, the aim of this study was to evaluate the gene expression and serum concentrations of IL-1 β , IL6 and TNF- α and insulin-like growth factor-1 (IGF-1), growth hormone (hGH), testosterone (T), and cortisol levels before and after a graded treadmill exercise to volitional exhaustion in female soccer players. The other objective was to analyze the relationships between cytokines and hormones and aerobic performance parameters of female soccer players.

Methods

Participants

Female soccer players (N=16), from the Ist division of the Polish soccer league participated in the study (age: 19.3 ± 2.3 years, body mass: 59.4 ± 7.5 kg, body height: 167 ± 1 cm, body mass index (BMI): 21.3 ± 2.1 kg/m²). Each participant had a minimum of 3 years training experience with a mean training status of approximately 6.7 ± 4.4 years. They were instructed to abstain from exercise, caffeine, alcohol, vitamins and any medicines' for 24-h prior to the exercise protocol. For the entire duration of the experiment, they were placed on a mixed isocaloric diet.

The study design was approved by the Bioethic Committee of the Medical University of Silesia (consent No. KNW/0022/KB1/122/I/09) and conformed to the standards set by the Declaration of Helsinki.

Maximal graded exercise test

All athletes performed an progressive exercise test which allowed to determine maximal oxygen uptake (VO_{2max}) and maximal power output (P_{max}). The graded treadmill exercise test (HP/Cosmos-Pulsar, Germany) was performed to volitional exhaustion. The exercise protocol started at a speed of 6 km per hour, and was

increased every 3 min by 2 km per hour until a maximal individual load was reached. Oxygen uptake (VO_2), pulmonary minute ventilation (V_E), and heart rate (HR) were continuously monitored from the 6th minute before the onset of the test and during the exercise using a respiratory analyzer (Matalyzer 3B, Cortex, Germany) and a telemetric HR monitor (PE-3000 Sport-Tester, Polar Inc. Finland). Before the exercise protocol, body mass was recorded and the body mass index was calculated (InBody Data Management System, UK).

Biochemical analyses

Blood samples were collected from the cubital vein at three time points: 1) between 08.00 and 09.00 h at rest (pre-exercise), 2) immediately after the cessation of exercise (post-exercise), and 3) after 15 minutes of recovery (recovery). All serum samples were left to clot at room temperature for 30 min and centrifuged for 15 min at $1000 \times g$ and kept frozen at -80°C (for a period not longer than eight months, without repeated freezing) until analyses for concentration of IGF-1, IGFBP-3, hGH, T, freeT, C, TGF- β , and interleukins (IL-6 and IL-1 β) were performed. Blood samples were also collected in order to examine blood morphology and the gene expression of and IL-1 β , IL6, TNF- α . The whole blood intended for RNA isolation was frozen at liquid nitrogen temperature until use. The complete blood count was determined on the same day using ABX MICROS ES-60 hematology analyzer (HORIBA Medical). The isolated RNA (Chomczyński and Sacchi, 1987) was determined with a spectrophotometry procedure GeneQuant pro Amersham Biosciences ($\lambda = 260, 280$ and 320 nm). Gene expression was estimated using the RT-qPCR reaction in the Opticon™ DNA Engine Continuous Fluorescence Detector (MJ Research, Watertown, MA) under standard thermal conditions according to Qiagen's instructions. The *reaction mixture* contained the QuantiTect® probe RT-PCR kit with RT enzyme, total RNA from PBMC (peripheral blood mononuclear cells) and *ACTB* - β -actin (*Hs 99999903_mL*) or *GAPDH* - Glyceraldehyde-3-phosphate dehydrogenase (*Hs99999905_mL*) or *IL-1 β* (*Hs00174097_mL*) or *IL-6* (*Hs174131_mL*) or *TNF- α* (*Hs00174128_mL*) primers. The specificity of amplification was assessed based on the melting curve. The expression of the above mentioned genes for each

athlete was determined in triplicate.

The concentration of IL-1 β , IL-6, and TNF- α was measured in the serum using the Quantikine ELISA Kits at standard procedure (R&D Systems, Inc.). The IL-1 β , IL-6, and TNF- α detection limits were 1.0, 0.7, and 1.5 pg per 1 mL, respectively. The concentration of insulin-like growth factor-1 (IGF-1) was determined with the IGF1-RIACT kit (Cisbio, France) and growth hormone (hGH) levels were determined with the hGH-RIACT kit (Cisbio, France). The insulin-like growth factor-binding protein 3 (IGFBP-3) concentration was also assessed with the IRMA method using the IGFBP-3-IRMA-CT KIPB1014 kit (Biosource Europe SA Company, England). The concentrations of testosterone (T), free testosterone (freeT), and cortisol (C) in blood serum were determined by radioimmunoassay (RIA) using TESTO-CT-2 (Cisbio, France) and CORT-CT-2 (Cisbio, France) kits per manufacturer's instructions.

Blood lactate concentrations were determined using the Biosen C_line method (EKF Diagnostic GmbH, Germany); acid-base balance was also analyzed (RapidLab 348; Bayer Diagnostics, Germany). Changes in plasma volume were taken into account during the evaluations.

Statistical analysis

The statistical analysis of the obtained results were performed using Statistica 13.1 software (StatSoft, Inc. Tulsa, OK, USA). Data distribution was assessed with the Kolmogorov-Smirnov test for normality. The arithmetic mean and the standard deviation (SD) were calculated for age, training experience, VO_2 , $\text{VO}_{2\text{max}}$, HR, HR_{max} , V_E , P_{max} , BMI, cytokine protein and hormone concentrations. The data were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test when appropriate. Differences between the genes expression and their significance were calculated using REST software (Pfaffl et al., 2002) calculating $2^{-\Delta\Delta\text{Ct}}$. Pearson correlation coefficients were analyzed to determine the relationships between cytokines and the physiological variables of exercise performance. Spearman's rank correlation coefficient was used for non-normal distributed data (ie., gene expression). Statistical significance was set at $p < 0.05$.

Results

The physiological performance variables and the blood variables of female soccer players before and after the intense graded treadmill exercise are presented in Table 1.

Significant differences between pre-exercise and post-exercise WBC count ($p < 0.000$) and a tendency for higher blood HGB ($p = 0.054$) concentration were observed (Table 1).

The relative expression of *IL-1 β* , *IL-6*, and *TNF- α* genes (compared to housekeeping gene - *GAPDH*) calculated according to the time of the experiment (pre-exercise data was basal), is presented in Table 2. The ANOVA revealed a significant effect of exercise on serum *IL-6* concentrations ($F = 17.8$ $p = 0.001$), but not on *IL-1 β* and *TNF- α* ($p > 0.05$) levels. A significant increase was confirmed in post hoc analyses between the pre-exercise and recovery *IL-6* levels ($p = 0.005$) (Figure 1).

A significant effect of exercise on serum C levels ($F = 8.15$, $p < 0.001$) and T/C ratio ($F = 9.15$, $p < 0.001$) were found. Exercise significantly increased serum C levels immediately after exercise ($p < 0.02$) and during 15 min of recovery ($p < 0.001$) (Table 3). A significant increase was observed in post-exercise serum T/C ratio ($p < 0.001$) and hGH ($p < 0.000$) levels compared to pre-exercise levels. The ANOVA revealed a non-significant trend for higher IGF-1 ($p = 0.10$) and freeT ($p = 0.18$) levels after exercise compared to pre-exercise levels (Table 3).

As for the relationship between all of the obtained results, the post-exercise gene expression of *IL-1 β* was positively correlated with post-exercise *IL-6* ($R = 0.84$, $p = 0.000$). The recovery *IL-1 β* gene expression was in an inverse relation with the age of the players ($R = -0.56$, $p = 0.038$) and HR_{max} ($R = -0.67$, $p = 0.013$). The recovery *IL-6* was found to significantly correlate with the recovery *TNF- α* gene expression ($R = 0.65$, $p = 0.009$). Furthermore, post-exercise serum concentration of *IL-1 β* showed correlations with P_{max} (Figure 2 A) and BMI (Figure 2 B); the higher the intensity of maximal exercise (and BMI) of the players, the higher the serum *IL1 β* concentration. Similarly to gene expression relation, the recovery *IL1 β* serum concentration also inversely correlated with the age of the players (Figure 2 C). Pre-exercise serum *IL-6* concentration was inversely correlated with training experience of the female soccer players (Figure 2 D), ie., the longer the training period, the lower the pre-exercise serum level of *IL6*.

There were positive correlations between hGH levels and VO_{2max} ($R = 0.50$ $p = 0.05$) and a negative correlation between VO_{2max} and BMI ($R = -0.68$ $p = 0.01$). Post-exercise C and hGH correlated positively with IGF-1 levels ($R = 0.52$, $p = 0.05$ and $R = 0.60$, $p = 0.02$, respectively). Moreover, the recovery T/C ratio was in an inverse relation with IGF-1 levels. Immediately post-exercise freeT levels correlated negatively with serum *IL-1 β* levels ($R = -0.56$, $p = 0.03$). The absence of other correlations between hormones and cytokines were revealed in female soccer players.

Table 1
Physiological variables and blood variables in response of graded exercise of examined female soccer players (mean \pm SD).

Variables	Pre-exercise	Post-exercise	<i>p</i>
VO ₂ [mL kg ⁻¹ min ⁻¹]	0.39 \pm 0.09	43.8 \pm 5.0	0.000
VE _{max} [mL min ⁻¹]	11.10 \pm 2.29	92.0 \pm 10.8	0.000
HR [bpm]	91.63 \pm 17.96	195.07 \pm 7.61	0.000
WBC [10 ³ mm ⁻³]	6.69 \pm 1.29	11.24 \pm 2.04	0.000
RBC [10 ⁶ mm ⁻³]	4.54 \pm 0.22	4.63 \pm 0.24	0.070
HGB [g dL ⁻¹]	13.83 \pm 0.90	14.00 \pm 0.94	0.054
HCT [%]	40.84 \pm 2.75	41.74 \pm 2.68	0.059
LA [mmoL mL ⁻¹]	1.04 \pm 0.01	7.67 \pm 2.8	0.000
pH	7.41 \pm 0.03	7.30 \pm 0.9	0.010

VO₂ – oxygen uptake; VE-pulmonary minute ventilation; HR- heart rate; WBC – number of white blood cells; RBC- number of red blood cells; HGB-haemoglobin; HCT-haematocrit; LA-blood lactate concentration; *p* – significant differences between pre-exercise and post-exercise value.

Table 2
Transcriptional activity of IL1- β , IL-6 and TNF- α in peripheral blood
of the female soccer players in response to exercise.

Gene	Post-exercise			Recovery		
	Expression	SE	P	Expression	SE	P
IL1B	1.17	0.009-132.4	0.889	2.41	0.04-110.2	0.420
IL6	1.93	0.02-196.8	0.580	15.1	0.167-1745.2	0.030
TNFA	1.09	0.004-177.2	0.940	5.75	0.02-481.7	0.162

Post-exercise – immediately after finished exercise test vs. pre-exercise;
Recovery – 15 minutes after exercise test vs. post-exercise. SE – standard error interval;
P – probability; Expression of reference gene GAPDH = 1.00.

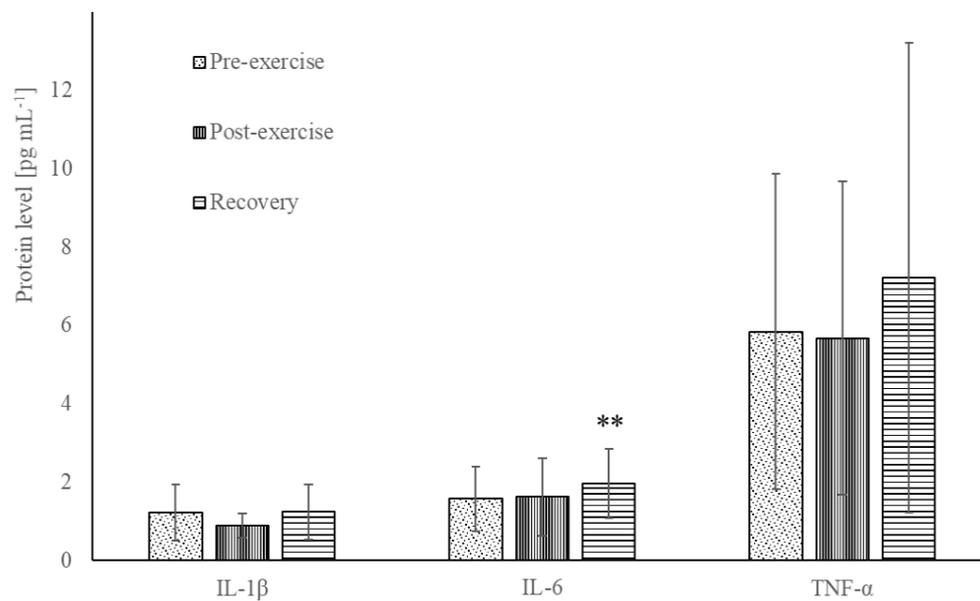


Figure 1

Cytokine concentrations in blood serum of the female soccer players (mean \pm SD) Pre-exercise - immediately before exercise; Post-exercise – immediately after exercise; recovery – 15 minutes after cessation of exercise; SD – standard deviation. ** $p < 0.005$ indicates statistically significant differences between pre-exercise and recovery values.

Table 3

Serum hormone concentrations in response to the graded exercise of female soccer players (mean ± SD).

Variables	Hormone kinetics			Effect of exercise		Post hoc		
	Pre-exercise (A)	Post-exercise (B)	Recovery (C)	F	p	Post hoc A vs B	Post hoc A vs C	Post hoc B vs C
T [nmol/l]	3.8 (1.7)	4.3 (1.5)	4.3 (1.4)	1.36	0.27	0.52	0.46	1.00
C [nmol/l]	457.0 (163.6)	571.0 (145.6)	609.2 (206.7)	8.15	0.001	0.02	0.001	1.00
T/C*100	0.7 (0.3)	1.0 (0.4)	0.8 (0.4)	9.15	0.001	0.001	1.00	0.01
HGH [μ lU/l]	16.1 (13.9)	52.5 (27.6)	45.1 (32.8)	16.29	0.000	0.000	0.000	0.85
IGF-1 [ng/ml]	236.6 (54.6)	250.4 (70.2)	263.7 (78.7)	2.51	0.10	0.78	0.10	0.84
IGFBP-3 [ng/ml]	2722.5 (979.0)	3033.7 (1255.0)	2573.0 (896.7)	1.44	0.25	0.81	1.00	0.32
freeT [pg/ml]	1.6 (0.8)	1.6 (0.7)	1.7 (0.7)	1.80	0.18	1.00	0.21	0.68

T - testosterone, C - Cortisol, hGH - human growth hormone, IGF-1 - insulin like growth hormone, IGFBP - 3 insulin like growth hormone binding protein -3.

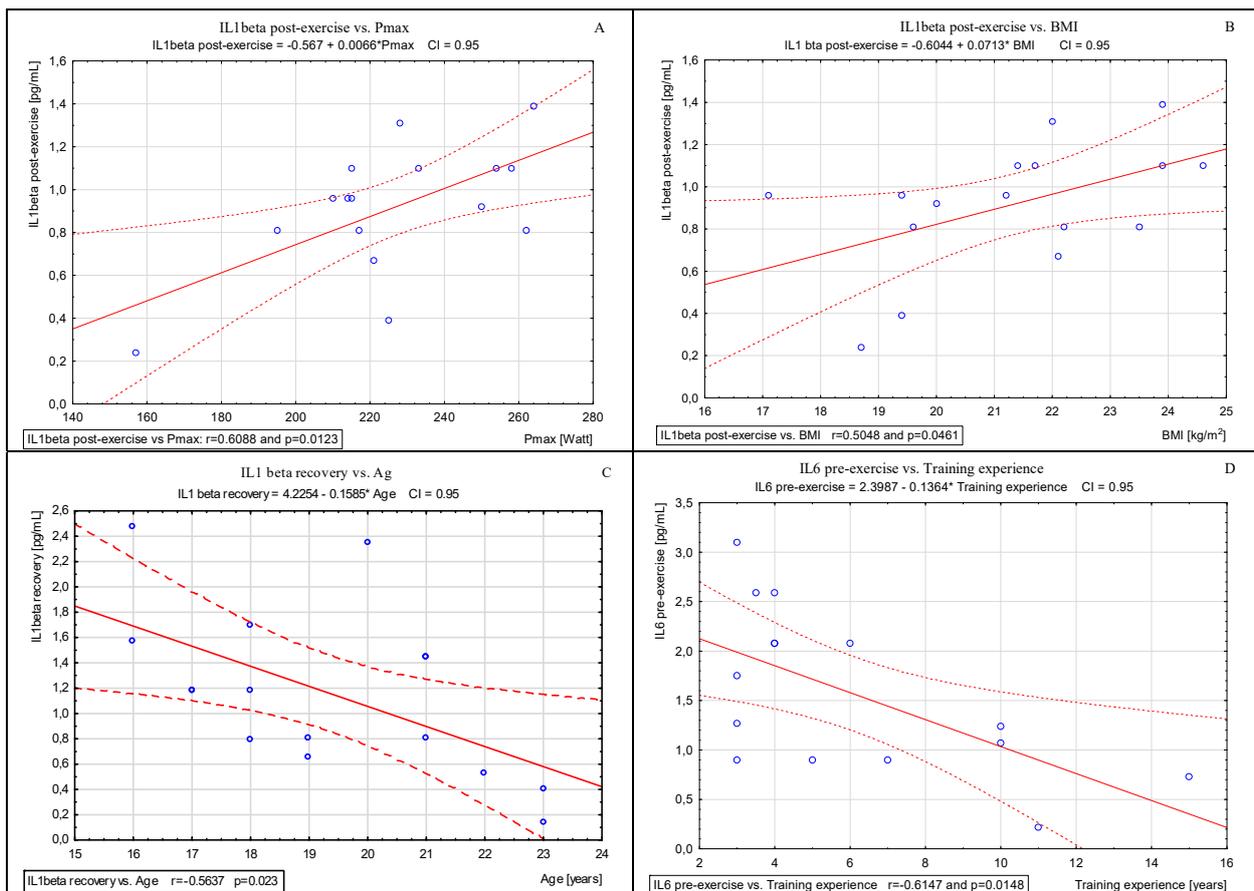


Figure 2

Correlation between serum concentration of IL-1 β post-exercise and maximal power (a) and body mass index (b); recovery IL-1 β and age (c), and pre-exercise IL-6 and training experience of the female soccer players (d).

Discussion

Our findings confirm previous reports that the strain of exercise elicited differences in IL-6 gene expression, as well as protein level during the recovery period after intense exercise of trained athletes. It is well-known that exercise-induced expression of IL-1 β and TNF- α is associated with higher transcriptional activity of IL-6 (Gjevestad et al., 2015). It should be stated that the intense treadmill exercise to volitional exhaustion elicited a time-dependent increase of IL-6 during recovery, but did not alter serum IL-1 β and TNF- α concentration. Previous studies support the findings that exercise which induces gene expression of inflammatory markers (acute phase cytokines) and their signaling pathways may constitute a sensitive tool to characterize the early effects of exercise on immune regulation (Pedersen and Hoffman-Goetz, 2000; Radom-Aizik et al., 2009) and adaptive processes during exercise (Ostrowski et al., 1999; Gillum et al., 2011; Roli et al., 2018). Physical stress causes the deregulation of muscle homeostasis and fiber integrity. The immune system is also directly involved in several repair and regeneration processes after exercise. IL-1 β and TNF- α are secreted during injuries and infections, whereas interleukin 6 is generally synthesized after the initial synthesis of these cytokines e.g. as a result of the contracting muscles (Walsh et al., 2011; Reihmane and Dela, 2014). IL-6 also contributes to metabolic substrate availability and may be involved in an increase of central fatigue, overtraining and hormone responses to training (Smith, 2000; deRocha et al., 2019).

There are limited data regarding the mechanism that determines the differences in women's immune system. To our knowledge, this study is the first to examine gene expression and protein concentrations of the pro-inflammatory cytokines in elite female soccer players in response to endocrine factors during exercise. In this study, no significant changes in IL-1 β and TNF- α gene expression and protein concentrations were observed in response to the intense exercise. Thus, our results may indicate no signs of inflammation or tissue damage. Similar to our study, Ullum et al. (1994) did not observe any effect of exercise on IL-1 β and TNF- α gene expression in healthy moderately trained

individuals. A significant increase of IL-6 levels and leukocyte count with no differences in IL-1 β , and TNF- α levels and gene expression of IL-1 β and TNF- α in blood mononuclear cells were also reported (Moldoveanu et al., 2000; deRocha et al., 2019).

In our findings a negative correlation was observed between the level of pre-exercise IL-6 and training status. A number of factors such as individual genetic variability, different exercise protocols, age and gender of the athletes, as well as other lifestyle factors influence the effects of post-exercise inflammatory processes (Abbasi et al., 2013; Andersson et al., 2010; Radom-Aizik et al., 2009). Our results confirm that there exists a significant positive correlation between exercise intensity and IL-1 β levels. Higher transcriptional activity of IL-1 β may be associated with the exercise-induced increase in gene and protein level of IL-6. Our results showed that training experience of the players has a significant effect on post-exercise serum IL-6 concentration; the longer the training experience the lower the serum IL-6. However, post-exercise IL-1 β level increases in response to higher body mass index and lower post-exercise free testosterone levels. It should be concluded that these relations may depend on the gender or individual performance level and need a more extensive explanation.

In our study, the female soccer players demonstrated higher serum cortisol levels recorded in response to the graded exercise and significantly higher serum T/C ratio compared to pre-exercise levels. Cortisol is the main human glucocorticoid secreted from the adrenal cortex in response to physical and psychological stress (Meckel et al., 2011). Exercise induced cortisol may be responsible for anti-inflammatory properties and inhibition of amino acid uptake by skeletal muscle, lower protein synthesis and catabolic effects of exercise (Popovic et al., 2019). Intensive exercise and training represents a strong stimulus for cortisol response and may induce lower testosterone secretion. On the contrary, an increased concentration of testosterone is vital for exercise metabolism (e.g.: the synthesis of structural proteins, protects them against catabolism, and supports the restoration of glycogen stores after exercise (Anderson et al., 2016). At rest, approximately 99% of total testosterone is bound with sex hormone-binding

globulin and only 1% of T is free. Therefore, the increased concentration of freeT levels in response to exercise may stimulate muscle hypertrophy in training related conditions. While cortisol secretion can be used as an indicator of physical stress and a catabolic marker, the testosterone to cortisol ratio (T/C) may represent a physiological indicator of anabolic/catabolic disbalance, fatigue and overreaching in athletes (Lac et al., 2000; Olivera et al., 2009).

Our study revealed higher post-exercise serum T/C ratio in our female soccer players compared to pre-exercise values, suggesting that T may be involved in beneficial adaptation to exercise and muscle hypertrophy. The positive correlation observed between total testosterone and freeT and a negative correlation between freeT and IL1 β may suggests that the hypertrophic effect observed in our athletes was stimulated by higher affinity of free testosterone lower this cytokine expression. This could be supported by previous data which have shown that endurance training may result in up-regulation of androgen receptors, thereby increasing the effectiveness of testosterone action (Sato et al., 2016; Zeng et al., 2017).

Although previous studies identified a significant stimulatory effect of exercise (Schwarz et al., 1996; Gregory et al., 2013; Hackney et al.,

2016) and training of female athletes (Roli et al., 2018) on serum IGF-1 levels, our data showed no significant differences in either IGF-1 and IGFBP-3 in response to graded exercise. In the present study, post-exercise cortisol levels seem to stimulate higher IGF-1 levels, whereas higher T/C levels correspond to lower hGH and IGF-1 secretion.

There are limited data regarding the mechanism that determines the anabolic hormone function in response to exercise. It seems that exercise induces a lower concentration of cortisol and higher testosterone bioavailability in female athletes with a greater performance capacity.

Conclusions

In summary, the exercise-induced inflammatory reaction was only reflected by a significant increase in gene and protein level of IL6. A novelty of this study is the finding that gene expression and serum concentrations of IL1 β are inversely associated with age and testosterone availability in female soccer players. Changes in cytokine levels and their mutual relationships may be indicative of physiological adaptation to intense exercise and its benefits to health, and compensatory attempt to restore immune homeostasis in trained female soccer players.

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Corresponding author:**Aleksandra Żebrowska**

Institute of Sport Sciences, Academy of Physical Education,
Mikołowska Street 72a, 40-065 Katowice, Poland
Phone: +48 608418581
E-mail: a.zebrowska@awf.katowice.pl