

**ACTH, CORTISOL AND IL-6 LEVELS IN ATHLETES FOLLOWING
MAGNESIUM SUPPLEMENTATION**NIVOI ACTH, KORTIZOLA I IL-6 KOD SPORTISTA
NAKON SUPLEMENTACIJE MAGNEZIJUMOMGordana Dmitrašinović¹, Vesna Pešić², Dušanka Stanić², Bosiljka Plečaš-Solarović²,
Marijana Dajak³, Svetlana Ignjatović^{1,3}¹Department of Medical Biochemistry, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia²Department of Physiology, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia³Center for Medical Biochemistry, Clinical Center of Serbia, Belgrade, Serbia**Summary**

Background: Physical exercise activates the hypothalamo-pituitary-adrenal (HPA) axis and induces the body's inflammatory response. Due to contemporary dietary habits and increased energy expenditure, athletes are susceptible to depletion of magnesium ions. The aim of our study was to investigate, through assessment of plasma ACTH, serum IL-6, and salivary/serum cortisol levels, if chronic magnesium supplementation might reduce damaging stress effects in amateur rugby players.

Methods: Rugby players (N=23) were randomly assigned to intervention and control group. Basal samples were collected before intervention group started a 4-week-long supplementation with magnesium (500 mg Mg/d). Blood and saliva sampling were done a day before the match (Day-1), on the morning of competition (Game), and during a six-day-long recovery period (Day1, Day3 and Day6). ACTH, serum/salivary cortisol, IL-6 and total/differential leukocytes counts were determined at each time point.

Results: There was a statistically significant increase in ACTH concentration in intervention group compared to control group, while reductions in cortisol concentrations between the two groups were the greatest at Day-1 ($p<0.01$) and at the day of competition (Game) ($p<0.01$). Our results revealed that magnesium completely abolished the increase in IL-6 level noted in control group on Day1

Kratak sadržaj

Uvod: Fizičko vežbanje aktivira hipotalamo-hipofizno-nadbubrežnu (HPA) osovinu i indukuje inflamatorni odgovor organizma. Sportisti su, zbog savremenih navika u ishrani i povećanog utroška energije, posebno podložni nastanku deplecije jona magnezijuma. Cilj naše studije bio je da se, praćenjem nivoa ACTH, serumskog IL-6 i nivoa salivarnog/serumskog kortizola, ispita da li hronična suplementacija magnezijumom može da smanji štetne efekte stresa kod amaterskih ragbi igrača.

Metode: Ragbi igrači (N=23) nasumice su podeljeni u interventnu i kontrolnu grupu. Bazalni uzorci su prikupljeni pre nego što je interventna grupa započela suplementaciju magnezijumom (500 mg Mg/dan). Krv i saliva su uzorkovani dan pre utakmice (Day-1), na dan takmičenja (Game) i tokom 6 dana perioda oporavka (Day1, Day3 and Day6). ACTH, serumski/salivarni kortizol, IL-6 kao i ukupan/diferencijalni broj leukocita su određeni u svakoj tački vremena.

Rezultati: Uočen je statistički značajan porast u koncentraciji ACTH u interventnoj grupi u poređenju sa kontrolnom grupom, dok je sniženje nivoa kortizola, između ispitivanih grupa bilo najveće dan pre utakmice (Day-1) ($p<0,01$) i na dan utakmice (Game) ($p<0,01$). Naši rezultati su pokazali da je magnezijum u potpunosti poništio porast IL-6 koji je primećen u kontrolnoj grupi prvog (Day1) i trećeg dana (Day3) posle utakmice u odnosu na dan pre utakmice

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List of abbreviations: ACTH, adrenocorticotrophic hormone; ANOVA, analysis of variance; CBG, cortisol binding globulin; CLIA, chemiluminescent immunoassay; ECLIA, electrochemiluminescence method; Cser, serum cortisol; Csal, salivary cortisol; Csal/Cser index, salivary cortisol/serum cortisol index; Day-1, day before the competition; Day1, first day after the game; Day3, third day after the game; Day6, sixth day after the game; Game, the day of competition; HPA, hypothalamo-pituitary-adrenal; IL-6, interleukin 6; Mg, magnesium; %LY, percentage of lymphocytes; %NE, percentage of neutrophils; WBC, white blood cells.

and Day3 vs. Day-1 ($p < 0.01$) and also diminished the rise in neutrophil/lymphocyte ratio in intervention group vs. control group ($p < 0.01$).

Conclusions: These results suggest the possibly important influence magnesium supplementation might have on the change of parameters of HPA axis activity and reduction of immune response activation following strenuous physical exercise such as a rugby game.

Keywords: ACTH, amateur rugby, IL-6, magnesium supplementation, serum/saliva cortisol

Introduction

For physically active persons, who have an increased demand for energy expenditure, a balanced diet with proper dietary intake of micronutrients is of particular importance. Magnesium (Mg) ions have numerous roles in energy metabolism, as a cofactor of more than 300 enzymes, and need to be available in adequate amounts in the body, in order to maintain the proper performance level of athletes (1). Magnesium is a physiological regulator of membrane stability and is involved in neuromuscular transmission, immune, hormonal and cardiovascular function (2). In case of insufficient dietary intake, athletes are very sensitive to marginal magnesium deficiency during high intensity exercises, because of the disbalance in magnesium homeostasis caused by increased magnesium loss through urine and sweat (3). A rugby match represents a very intensive form of exercise which, as a form of physical stressor, can lead to activation of the hypothalamo-pituitary-adrenal (HPA) axis and the sympathetic nervous system, thus inducing changes in the concentration of different hormones as well as interleukin 6 (IL-6). It is well documented that IL-6, whose synthesis is a part of normal physiological response to exercise (4), modulates stress response and has the ability to activate the HPA axis at its various levels: through stimulation of cortisol release from the adrenal glands, and through its influence on the hypothalamus, stimulating the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland (5). In addition, prolonged intensive exercise can induce the body's specific inflammatory response followed by transitory leukocytosis and change in the leukocytes differential count (6). One of the earlier studies (7) showed that the neutrophil/lymphocyte ratio, which has been presumed to be an excellent marker of the systemic inflammatory response (8), increases after prolonged intensive exercise.

Several studies in magnesium depleted animals revealed that magnesium deficiency leads to increased inflammation (9) and change in the activity of HPA axis. Laarakker et al. (10) reported significant correlation between several anxiety-related behavioral parameters and plasma and brain magnesium levels.

Effects of magnesium supplementation in athletes were usually assessed with respect to exercise-

(Day-1) ($p < 0,01$) i doveo do sniženja odnosa između neutrofila i limfocita u interventnoj grupi u odnosu na kontrolnu ($p < 0,01$).

Zaključak: Ovi rezultati ukazuju na potencijalno značajan uticaj koji suplementacija magnezijumom može imati na parametre aktivnosti HPA osovine i na smanjenje aktivacije imunog odgovora nakon napornog fizičkog vežbanja kao što je ragbi utakmica.

Ključne reči: ACTH, amaterski ragbi, IL-6, suplementacija magnezijumom, serumski/salivarni kortizol

induced magnesium redistribution and its effect on physical strength performance (11–14). A limited number of studies investigated the influence of magnesium supplementation on hormonal response in trained athletes, especially its influence on the HPA axis activity parameters (15) as well as on the white blood cell (WBC) count and leukocytes percentage profile (16), but to our knowledge, none of them were done over a six-day-long period in amateur rugby players.

Having all the above-mentioned in mind, the aim of our study was to examine whether the changes of HPA axis activity parameters before a rugby match and during 6 days of the post-competition period were influenced by a 4-week-long Mg supplementation. Apart from following the changes in the plasma ACTH levels and salivary/serum cortisol, we also examined the changes in serum IL-6 level and WBC percentage profile.

Methods

Subjects

Players of an amateur rugby team ($N=23$) gave their written consent for participation in the study, after they had been fully informed of all experimental procedures. The study protocol has been approved by the Ethical Committee for Clinical Trials of the University of Belgrade, Faculty of Pharmacy. Participants were randomly assigned to intervention ($N=13$) and control ($N=10$) group, so that both types of players (forwards and backs) were well represented in both groups. All of them had trainings three to four times a week, for 2 hours, and a rugby match each Sunday in the early afternoon, starting at 14 h. Basic anthropometric data of both the control and intervention group of rugby players are given in *Table 1*.

Sample collection and analyses

Schedule of sampling is given in *Figure 1*. Before intervention, basal blood and saliva samples were collected a day before the match (e.g. Saturday), just one month after the beginning of the competing season. Players were randomly assigned to intervention ($N=13$) and control group ($N=10$).

During the next 28 days, players allocated to intervention group received 500 mg of magnesium, divided in two doses, twice a day with a 12 h interval between doses of 250 mg (Magnesium 250 mg®, Natural Wealth®, NBTY Inc.). They received daily text message reminders to consume their tablets and were asked to avoid intense consumption of food that typically contains high levels of Mg ions (such as cereals, whole grain breads) as well as not to take any other multivitamin supplements. On the first day after 4 weeks of supplementation (29th day of experiment) blood and saliva sampling started. That was a day before the match (Day-1). The match was played in the early afternoon (14 h). The blood and saliva samples were also collected in the morning before the match (Game), the next day after the match (Day1), on the third day (Day3) and the sixth day after the game (Day6), thus covering the six-day-long recovery period.

Table 1 Basic anthropometric data for control and intervention (Mg supplemented) group.

| | Control group | Intervention group |
|--------------------------|---------------|--------------------|
| n | 10 | 13 |
| Age (years) | 22.9 (1.18) | 23.6 (1.40) |
| Height (cm) | 182.0 (2.13) | 180.7 (1.65) |
| Body weight (kg) | 84.7 (1.87) | 85.1 (3.66) |
| BMI (kg/m ²) | 25.58 (0.430) | 25.97 (0.806) |

Values are shown as mean (SEM).

Blood and saliva samples were always collected in the morning, after 12 h overnight fast. Blood samples were obtained by antecubital venepuncture into two EDTA vacutainer tubes and one serum vacutainer tube with gel (Vacutainer; Becton, Dickinson and Company, NYSE, USA), according to standardized protocol. Blood samples collected in one of the EDTA vacutainer tubes were used for determination of hematological parameters. The other lavender top (EDTA) tube was put immediately on ice and cooled centrifuge was used for the immediate separation of plasma from cells. Serum tubes were centrifuged at room temperature (3000 rpm for 15 min). Both plasma and serum were aliquoted and stored in duplicates at -20 °C, for subsequent analyses. Two sponges (BD Visispear, Beaver-Visitec International, USA) per subject were used for saliva collection. After 60–90 seconds of saliva collection, sponges were placed in a conical tube with cap and centrifuged to extract saliva. Within 2 hours after collection, saliva samples were frozen and stored in duplicates at -20 °C until required.

Serum cortisol and IL-6 were measured using the CLIA method on an Access® 2 analyzer (Beckman Coulter, Inc., Brea, USA). According to manufacturer's instructions, the lower limits of detection of the assays were 11 nmol/L and 0.5 pg/mL for cortisol and IL-6, respectively. The total imprecision coefficient of variation for cortisol assay was 6.0% at 664.9 nmol/L while IL-6 assay had total imprecision < 12.0% at concentrations greater than 2 pg/mL. Plasma levels of ACTH were determined with the ECLIA method on an Elecsys analyzer (Roche Diagnostics GmbH, Mannheim, Germany) with intra- and

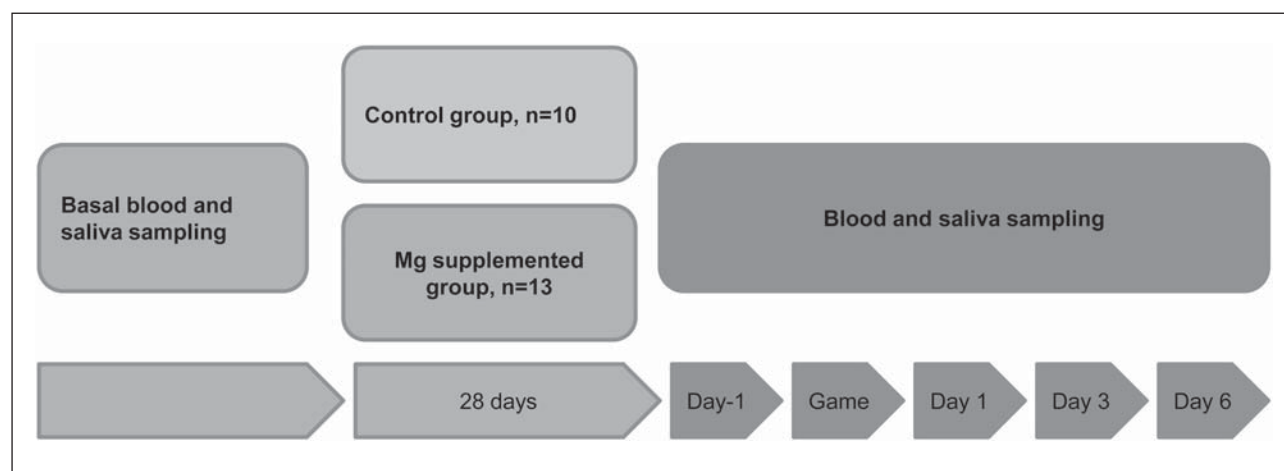


Figure 1 Algorithm of the study.

Before intervention, basal blood and saliva samples were collected on the day before the match. Players were randomly assigned to intervention (N=13) and control (N=10) group. Those allocated to the intervention group received during the next 28 days 500 mg Mg/day, divided in two equal doses, twice a day with a 12 h time interval between doses. On the first day after 4 weeks of supplementation (29th day of experiment) which was a day before match (Day-1), blood and saliva were collected. Biological fluid sampling was also done on the day of the competition (Game), on the next day (Day1), the third day (Day3) and the sixth day after the game (Day6).

inter-assay CV < 2.9% and < 5.4%, respectively, at 1.08 pmol/L. Salivary cortisol was analyzed on the same instrument with an assay whose limit of detection was 0.50 nmol/L and intra-assay imprecision for salivary concentrations of 4.68 nmol/L and 19.8 mol/L was 6.1% and 2.8%, respectively. Analyses of total white blood cells count (WBC) and WBC differential were determined at Day-1, Day1 and Day6 using a Beckman Coulter ®LH750 analyzer.

Statistical analysis

T-test was used for the comparison of the basal levels of all measured parameters between intervention and control group, as well as for the comparisons of the basal values and the levels of measured parameters at the day before the match (Day-1), for each individual group. Two-way analysis of variance (ANOVA) was used to analyze the effect that Mg supplementation might have on the dynamic of the change of all measured parameters, at specified time points (Day-1, Game, Day1, Day3 and Day6); if a significant F value was obtained, simple main effect was run separately for treatment and time, using Boniferoni *post hoc* test for multiple comparisons and $p < 0.05$ was considered to be statistically significant.

If the interaction effect was not statistically significant, an analysis of main effect for each independent variable was preformed. Prior to statistical analyses, log transformation was applied to data for which the assumption of normality had been violated. The data were analyzed using the SPSS version 21.0 software and presented as means (SEM).

Results

After the participants were randomly assigned to intervention (N=13) and control (N=10) group, the basal concentrations of all measured parameters were compared using T-test. There was no statistically significant difference ($p > 0.05$) in the concentrations of measured parameters between groups before the intervention group started with supplementation, thus the basal values of all participants are shown in *Figures* and *Table* as unique basal values.

Two-way ANOVA did not show the statistically significant interaction between the effect of Mg supplementation and time on ACTH concentration ($F_{(4,105)} = 1.695$, $p = 0.157$) even though a different pattern of ACTH level changes in supplemented and non-supplemented athletes could be seen in *Figure 2*.

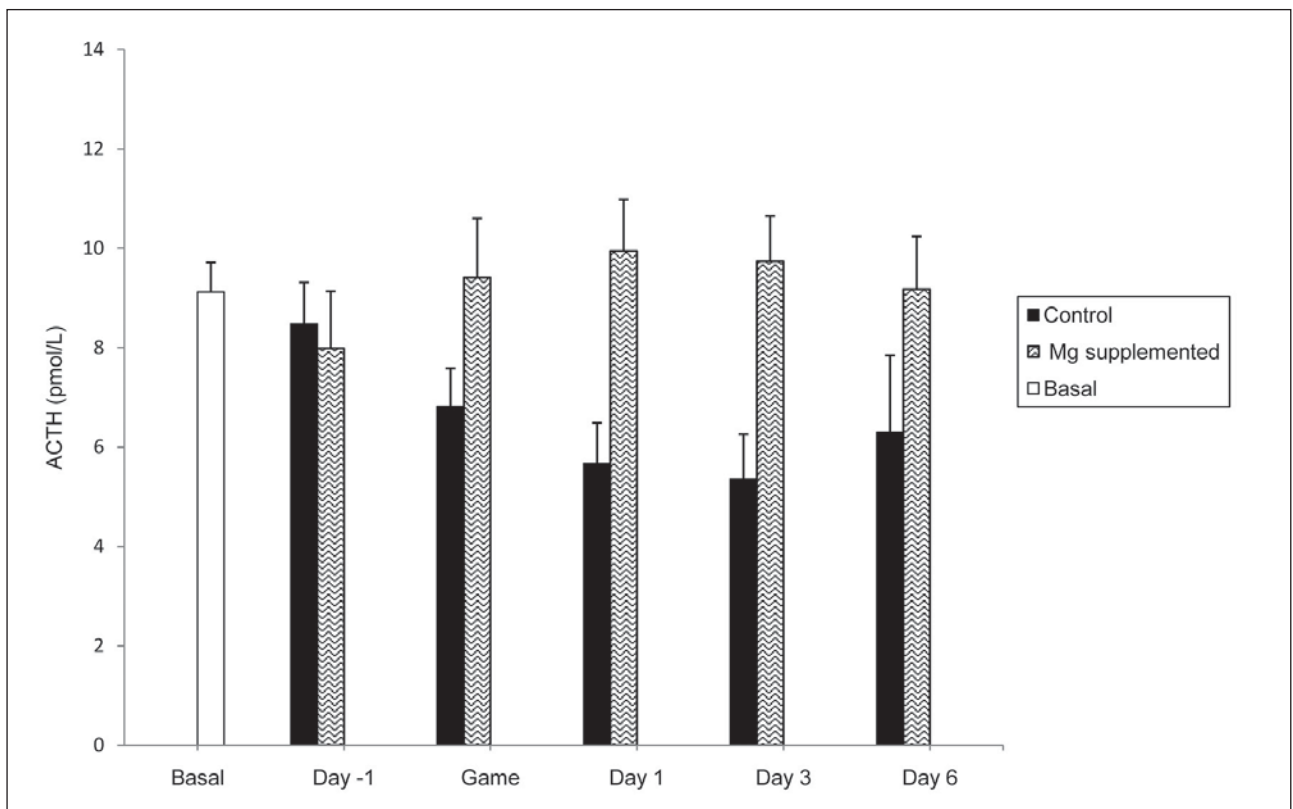


Figure 2 Mg influences ACTH pattern of change during post-competition period.

Changes in ACTH level across sample points, control (black bars) and Mg supplemented group (grey bars), with basal values (white bar).

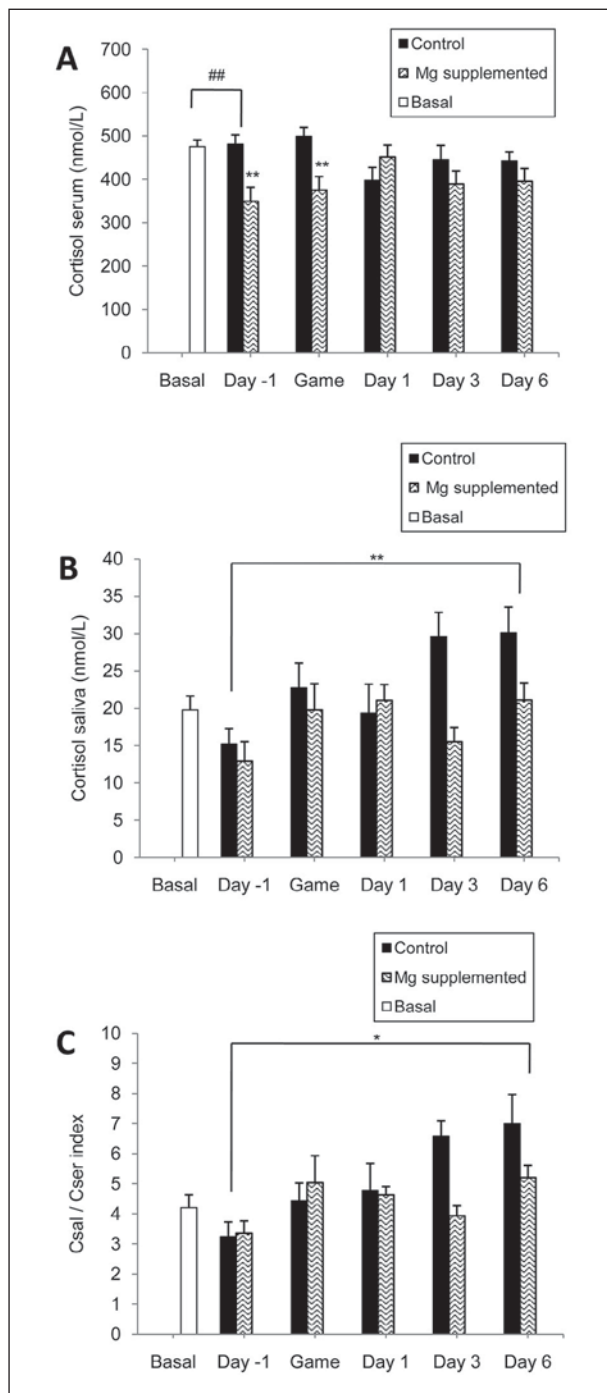


Figure 3 The change in serum and salivary cortisol levels. Changes in cortisol levels across sample points, the control group (black bars) and Mg supplemented group (grey bars), with basal values (white bar). (A) Changes in serum cortisol levels: ** $p < 0.01$ values for corresponding time points for control group vs. Mg supplemented. ## $p < 0.01$ values for level of serum cortisol in Mg supplemented rugby players on the day before the match (Day-1) vs. basal cortisol level. (B) Changes in salivary cortisol levels: ** $p < 0.01$ the main effect of time showed statistically significant difference in mean salivary cortisol concentration between Day6 and Day-1. (C) Changes in calculated Csal/Cser index: * $p < 0.05$ the main effect of time showed statistically significant difference in mean Csal/Cser index between Day6 and Day-1.

Opposite to the main effect of time, the main effect of treatment showed a statistically significant difference in ACTH concentration between the intervention and the control group ($F_{(1,105)} = 16.207$, $p < 0.001$). There was a statistically significant increase of 2.73 (6.79) pmol/L in ACTH concentration in the intervention group compared to the control group. There was no significant difference between the mean basal and Day-1 ACTH concentration both for non-supplemented ($t_{18.663} = 0.620$, $p > 0.05$) and supplemented athletes ($t_{18.663} = 0.873$, $p > 0.05$).

Our results showed a statistically significant interaction between the effect of Mg supplementation and time on the concentration of serum cortisol ($F_{(4,105)} = 3.261$, $p = 0.015$). Taking into consideration the effect of magnesium administration, different patterns of cortisol response to intensive physical exercise are shown in Figure 3A. The simple main effect of treatment showed statistically significant decrease in serum cortisol (Cser) concentration in the intervention group compared to the control group ($F_{(1,105)} = 10.923$, $p = 0.001$). *Post hoc* analyses with Boniffferoni adjustment revealed that these reductions in the concentration between the two groups were the greatest at Day-1 ($p < 0.01$) and at the day of the competition (Game) ($p < 0.01$). In the Mg-supplemented athletes, the level of serum cortisol was considerably reduced at the day before the game (Day-1) compared to its basal level ($p < 0.01$), while for the control group no statistically significant difference between basal and Day-1 levels was observed. Even though two-way ANOVA has not shown a statistically significant interaction between the effect of Mg supplementation and time on the concentration of salivary cortisol ($F_{(4,105)} = 2.301$, $p = 0.064$) as it could be seen in Figure 3B, the main effect of treatment showed a statistically significant difference in salivary cortisol (Csal) concentration between the two groups ($F_{(1,105)} = 8.533$, $p = 0.004$). A statistically significant decrease of 5.34 (1.83) nmol/L in the salivary cortisol concentration was noticed in the intervention group compared to the control group. The main effect of time showed a statistically significant difference in the mean salivary cortisol concentration between Day6 and Day-1 ($p < 0.01$). There was no statistically significant difference between the mean basal and Day-1 levels of salivary cortisol for neither the control nor the intervention group.

Furthermore, when the Csal/Cser index was calculated, it showed increase through all time points in the control group (Figure 3C). There was no statistically significant interaction between the effect of Mg supplementation and time on Csal/Cser index ($F_{(4,105)} = 1.575$, $p = 0.186$). Similarly to mean salivary cortisol concentration, the main effect of time showed a statistically significant difference in the mean Csal/Cser index between Day6 and Day-1 ($p < 0.05$). In addition, there was no statistically significant difference between basal and Day-1 values of

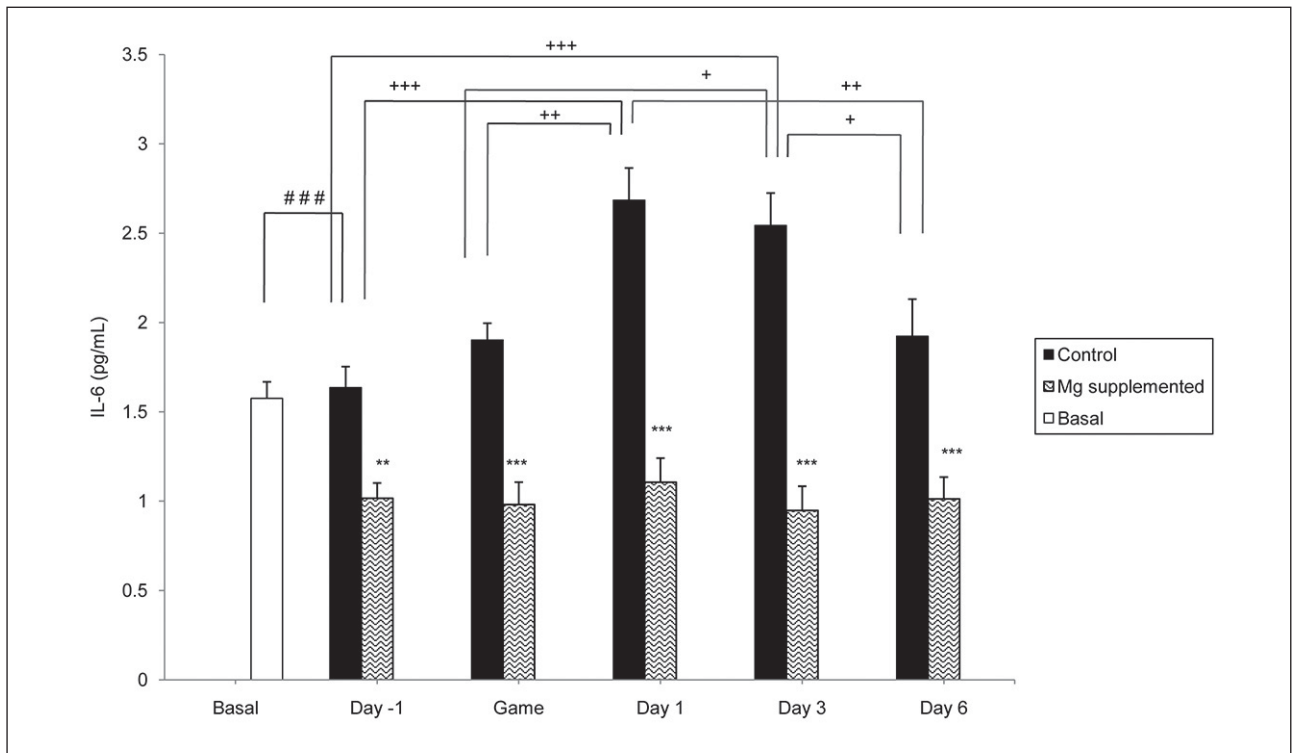


Figure 4 Mg supplementation significantly influences IL-6 serum level in rugby players.

Changes in serum IL-6 level across sample points, control (black bars) and Mg supplemented group (grey bars), with basal values (white bar). ** $p < 0.01$ and *** $p < 0.001$ values for corresponding time points for control group vs. Mg supplemented. + $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$ in the control group for different time points; ### $p < 0.01$ values for level of IL-6 in Mg supplemented rugby players on the day before the match (Day-1) vs. basal IL-6 level.

Table II White blood cells in the control group and Mg supplemented group of amateur rugby players.

| Variable | Basal (both groups) | Day-1 | | Day1 | | Day6 | |
|---------------------------------|------------------------|--------------|--------------------------|--------------------------|---------------------------|--------------------------|--------------------------|
| | | Control | Mg supplemented | Control | Mg supplemented | Control | Mg supplemented |
| Total WBC, $\times 10^9 L^{-1}$ | 7.45 (0.26) | 7.60 (0.27) | 6.42 (0.34) ^a | 6.73 (0.22) | 6.29 (0.28) | 6.65 (0.28) | 6.94 (0.26) |
| Neutrophils, % | 51.56 (1.61) | 51.83 (1.79) | 52.55 (1.28) | 56.81 (1.64) | 50.49 (1.38) ^a | 52.77 (1.70) | 53.70 (1.72) |
| Lymphocytes, % | 34.37 (1.54) | 33.85 (1.86) | 33.75 (1.19) | 29.72 (1.15) | 35.98 (1.21) ^a | 32.76 (1.23) | 35.72 (1.66) |
| Monocytes, % | 10.18 (0.41) | 10.47 (0.65) | 8.98 (0.48) ^a | 10.35 (0.91) | 9.38 (0.48) | 10.32 (0.92) | 6.83 (0.43) |
| Basophils, % | 0.83 (0.08) | 0.73 (0.06) | 0.43 (0.08) ^a | 0.47 (0.06) ^b | 0.45 (0.04) | 0.81 (0.04) ^c | 0.37 (0.06) ^a |
| Eosinophils, % | 3.07 (0.34) | 3.12 (0.47) | 4.29 (0.78) | 2.65(0.39) | 3.71 (0.66) | 3.13 (0.52) | 3.38 (0.64) |

Values are shown as mean (SEM). Changes in total WBC and WBC differential are determined on the day before the competition (Day-1), 24 hours after the competition (Day1) and on the sixth day of post-competition period (Day6). ^a $p < 0.05$ compared to the same point of time in the control group, ^b $p < 0.05$ compared to Day-1 in the control group, ^c $p < 0.05$ compared to Day1 in the control group.

Csal/Cser index for neither the control nor the intervention group.

Two-way ANOVA showed a statistically significant interaction between the effect of Mg supplementation and time on the concentration of IL-6 ($F_{(4,105)} = 4.896$, $p < 0.001$). The effect of the rugby match on IL-6 level changes was noted only in control group which was revealed by the simple main effect of time that showed a statistically significant difference ($F_{(1,105)} = 9.195$, $p < 0.001$) in the mean IL-6 concentration between the different days. The simple main effect of treatment showed a statistically significant decrease in IL-6 concentration in supplemented compared to non-supplemented athletes at each time point ($p < 0.01$) (Figure 4). There was a statistically significant difference between the mean basal and Day-1 levels of IL-6 for the Mg-supplemented group ($t_{32.297} = 4.424$, $p < 0.001$).

Two-way ANOVA was also used to analyze the effect that Mg supplementation might have on the dynamic of the change of hematological parameters, at specified time points (Day-1, Day1 and Day6). Our study showed that after four weeks of Mg administration, total WBC count on Day-1 was significantly lower ($p < 0.05$) in the Mg-supplemented group compared to the control, and changes in the differential formula were noticed as well (Table II). A statistically significant interaction between the effect of Mg supplementation and time on the percentage of neutrophils (%NE) ($F_{(2,63)} = 3.359$, $p = 0.041$) was shown. In control group, 24 h hours following the competition, an increase in the percentage of neutrophils (%NE), and decrease in the percentage of lymphocytes (%LY) were seen, thus resulting in a high NE/LY ratio. As for the Mg supplemented group, the main differences were found 24 hours after the competition (Day1): the simple main effect of treatment showed a statistically significant decrease of almost 12% in %NE, while the main effect of treatment showed a statistically significant increase of 9% in %LY, compared to their values measured in the control group, which caused a corresponding switch in the NE/LY ratio.

Discussion

The present study demonstrated that four-week-long Mg supplementation in amateur rugby players altered the pattern of change of ACTH and cortisol not only during the recovery period after a rugby match, but also influencing the levels of these hormones before the game. Furthermore, significant reduction of IL-6 levels in the supplemented rugby players and absence of the sharp rise of NE/LY ratio after the rugby match were also noted.

Cortisol level, together with ACTH level, was reduced after a rugby match in the control group. These results are in accordance with the studies conducted on rugby players (17) and weight lifters (18),

where immediately after strenuous physical activity, levels of these hormones were augmented and within several hours they were lower than their basal levels. According to the results obtained in our study, Mg supplementation changed this pattern. The most significant difference was the absence of anticipatory increase in cortisol level before the rugby match after Mg supplementation, which was obvious in the control group. These findings point to the possibility that supplemented players were less susceptible to anxiety-like behavior before the match.

There are several lines of evidence that support the role of Mg in the stress response system and anxiety-like behavior, but the exact mechanisms underlying these effects are not so clear yet. It was shown that Mg ion has N-methyl-D-aspartate (NMDA)-antagonistic and gamma-aminobutyric acid (GABA)-agonistic properties (19) and, thus, its elevation could contribute to reducing the level of anxiety. On the other hand, it was demonstrated that Mg deficiency leads to anxiety-related behavior and HPA axis disbalance that could be surmounted by use of anxiolytic and antidepressant drugs (20). Results obtained after Mg supplementation are in accordance with one of the studies that analyzed the Mg supplementation influence on hormonal and immune response in trained athletes (15). In this study, effects of a 1-month-long exercise program and Mg supplementation (10 mg Mg/kg/day) on ACTH and cortisol levels in young tae-kwon-do and sedentary subjects were explored and it was demonstrated that the levels of ACTH and cortisol were significantly increased after magnesium supplementation in all subjects, both at rest and at exhaustion. Although the daily dose of the magnesium supplement used in our study was lower, similar to these findings, our results for ACTH after Mg supplementation revealed the increase in ACTH concentration compared to its level in the control group, especially during the post-competition period: Day1 (+75%), Day3 (+82%) and Day6 (+46%).

Magnesium supplementation affected serum cortisol levels most significantly before the game, thus pointing to magnesium's role in the reduction of stress-anticipating anxiety. Nevertheless, the effect on the level of salivary cortisol, which could be considered to represent the free hormone fraction (21), was the most evident at the end of the post-competition period. It is well known that only 3–10% of total circulating cortisol is in the free unbound state, whereas the majority of the remaining cortisol binds with high affinity to cortisol binding globulin (CBG), and some 10–15% with low affinity to albumin (22). The levels of CBG may be considerably altered during inflammatory response, since it is considered to be a 'negative acute-phase protein'. While the level of total cortisol after the competition stays almost constant in time, the increase of free cortisol at Day3 and Day6 could be explained through the possible decrease of CBG production, which altogether could be the cause of

high free/total cortisol ratio in the control group during the post-competition period.

Response of IL-6 level after a rugby match was significantly affected by one-month-long Mg supplementation: the rise in IL-6 level after a rugby match that was observed in the control group was completely absent in the Mg supplemented group. Studies conducted on different populations showed significant elevation of post-exercise IL-6 levels: both in athletes, especially after 24 hours after the game played (23), as well in physically active volunteers who were not accustomed to high-intensity exercise (24). Our results for rugby players before Mg supplementation corroborate these findings. One of our previous trials on a male student population showed that chronic magnesium supplementation caused decrease in serum cortisol level that was also accompanied by reduction in IL-6 level (25). There is a substantial amount of data pointing to the role of Mg in cytokine production as a part of HPA axis disturbance and/or immune response. Mainly, those studies showed that Mg deficiency contributes to the C-reactive protein rise (26), phagocyte activation and consequently to higher cytokine production (27). Sugimoto et al. (28) showed that MgSO₄ supplementation increases IκBα levels, leading to reduced NF-κB activation and decrease in IL-6 and TNF-α cytokine production both *in vitro* and *in vivo*.

The change in neutrophil/lymphocyte ratio, which might be a marker of subclinical inflammation (29), is an important finding of the present study. The greatest change was noticed 24 hours after the competition (Day1) following Mg supplementation when the percentage of neutrophils decreased, while the percentage of lymphocytes increased, compared to the values before Mg supplementation, leading to a reduction of nearly 36% in the NE/LY ratio. It was suggested that the rise in total leukocyte count, particularly of neutrophils, is a consequence of the inflammatory reaction induced by exercise (30) and is proportional to the intensity and duration of physical activity (31). Strenuous physical exercise may lead to oxidative stress, which brings damage to lymphocyte DNA and subsequent reduction in their number and function (32). The results of our study are in compliance with previous findings that intensive physical training can lead to reduction in basophil count, which can be the consequence of complete basophil degranulation (33). Following 4-week-long Mg administration, no significant change was observed in basophil count before and after the competition. It is proved that Mg diminishes susceptibility to oxidative stress and is important in DNA stabilization (34). These results, together with the decrease of IL-6 and percentage of monocytes and basophils, suggest that prolonged Mg supplementation may possibly act as an antiinflammatory agent.

The major limitation of our study is the relatively small number of players who participated in the investigation and the lack of strict control of dietary intake during the pretrial period. Despite these limitations, this is the first prospective observational study that examined the association between the activity of HPA axis parameters and magnesium supplementation in amateur rugby players. Further larger and more controlled studies are needed to closely identify the underlying molecular mechanism delineating the association between magnesium and immune system response in physically active persons.

Conclusion

The purpose of our study was to explore the impact of four-week-long Mg supplementation in amateur rugby players on the IL-6 level and HPA axis activity parameters before a rugby match and during the 6-day-long post-competition period. Our results showed an altered pattern of change of ACTH and cortisol levels in Mg-supplemented rugby players compared to the control group. The significant reduction of IL-6 level in supplemented rugby players and the absence of sharp neutrophils to lymphocytes ratio rise after the rugby match were also noted. These findings pointed to the significant role that magnesium could have in reducing immune response activation just after a strenuous physical exercise such as a rugby game.

Further investigations are needed to check if the downregulation of HPA axis activity by Mg in rugby players could leave more space for the display of other hormones, possibly adrenaline or testosterone, in defining pre- and post-match behavior, thus influencing the optimal recovery between demanding competitions such as rugby matches.

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Conflict of interest statement

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

References

1. Laires MJ, Monteiro C. Exercise, magnesium and immune function. *Magnes Res* 2008; 21: 92–6.
2. Lukaski HC. Magnesium, zinc, and chromium nutrition and physical activity. *Am J Clin Nutr* 2000; 72: Suppl 2: 585S–93S.
3. Nielsen FH, Lukaski HC. Update on the relationship between magnesium and exercise. *Magnes Res* 2006; 19: 180–9.
4. Anđelković M, Baralić I, Đorđević B, Kotur Stevuljević J, Radivojević N, Dikić N, Radojević Škodrić S, Stojković M. Hematological and biochemical parameters in elite soccer players during a competitive half season. *J Med Biochem* 2015; 34: 460–6.
5. Gleeson M. Immune function in sport and exercise. *J Appl Physiol* (1985) 2007; 103: 693–9.
6. Lippi G, Banfi G, Montagnana M, Salvagno GL, Schena F, Guidi GC. Acute variation of leukocytes counts following a half-marathon run. *Int J Lab Hematol* 2010; 32: 117–21.
7. Nieman DC, Simandle S, Henson DA, Warren BJ, Suttles J, Davis JM, et al. Lymphocyte proliferative response to 2.5 hours of running. *Int J Sports Med* 1995; 16: 404–9.
8. Guthrie GJ, Charles KA, Roxburgh CS, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil-lymphocyte ratio: experience in patients with cancer. *Crit Rev Oncol Hematol* 2013; 88: 218–30.
9. Rayssiguier Y, Libako P, Nowacki W, Rock E. Magnesium deficiency and metabolic syndrome: stress and inflammation may reflect calcium activation. *Magnes Res* 2010; 23: 73–80.
10. Laarakker MC, van Lith HA, Ohi F. Behavioral characterization of A/J and C57BL/6J mice using a multidimensional test: association between blood plasma and brain magnesium-ion concentration with anxiety. *Physiol Behav* 2011; 102: 205–19.
11. Santos DA, Matias CN, Monteiro CP, Silva AM, Rocha PM, Minderico CS, et al. Magnesium intake is associated with strength performance in elite basketball, handball and volleyball players. *Magnes Res* 2011; 24: 215–9.
12. Kass LS, Skinner P, Poeira F. A pilot study on the effects of magnesium supplementation with high and low habitual dietary magnesium intake on resting and recovery from aerobic and resistance exercise and systolic blood pressure. *J Sports Sci Med* 2013; 12: 144–50.
13. Veronese N, Berton L, Carraro S, Bolzetta F, De Rui M, Perissinotto E, et al. Effect of oral magnesium supplementation on physical performance in healthy elderly women involved in a weekly exercise program: a randomized controlled trial. *Am J Clin Nutr* 2014; 100: 974–81.
14. Setaro L, Santos-Silva PR, Nakano EY, Sales CH, Nunes N, Greve JM, et al. Magnesium status and the physical performance of volleyball players: effects of magnesium supplementation. *J Sports Sci* 2014; 32: 438–45.
15. Cinar V, Mogulkoc R, Baltaci AK, Polat Y. Adrenocorticotropic hormone and cortisol levels in athletes and sedentary subjects at rest and exhaustion: effects of magnesium supplementation. *Biol Trace Elem Res* 2008; 121: 215–20.
16. Mooren FC, Golf SW, Volker K. Effect of magnesium on granulocyte function and on the exercise induced inflammatory response. *Magnes Res* 2003; 16: 49–58.
17. Elloumi M, Maso F, Michaux O, Robert A, Lac G. Behaviour of saliva cortisol [C], testosterone [T] and the T/C ratio during a rugby match and during the post-competition recovery days. *Eur J Appl Physiol* 2003; 90: 23–8.
18. Passelergue P, Lac G. Saliva cortisol, testosterone and T/C ratio variations during a wrestling competition and during the post-competitive recovery period. *Int J Sports Med* 1999; 20: 109–13.
19. Murck H. Magnesium and affective disorders. *Nutr Neurosci* 2002; 5: 375–89.
20. Sartori SB, Whittle N, Hetzenauer A, Singewald N. Magnesium deficiency induces anxiety and HPA axis dysregulation: modulation by therapeutic drug treatment. *Neuropharmacology* 2012; 62: 304–12.
21. Aardal-Erickson E, Kalberg BE, Holm AC. Salivary cortisol – an alternative to serum cortisol determinations in dynamic function tests. *Clin Chem Lab Med* 1998; 36: 215–22.
22. Levine A, Zagoory-Sharon O, Feldman R, Lewis JG, Weller A. Measuring cortisol in human psychobiological studies. *Physiol Behav* 2007; 90: 43–53.
23. Cunniffe B, Hore AJ, Whitcombe DM, Jones KP, Baker JS, Davies B. Time course of changes in immunoenocrine markers following an international rugby game. *Eur J Appl Physiol* 2010; 108: 113–22.
24. Philippou A, Bogdanis G, Maridaki M, Halapas A, Sourla A, Koutsilieris M. Systemic cytokine response following exercise-induced muscle damage in humans. *Clin Chem Lab Med* 2009; 47: 777–82.
25. Zogović D, Pešić V, Dmitrašinović G, Dajak M, Plečaš B, Batinić B, Popović D, Ignjatović S. Pituitary-gonadal, pituitary-adrenocortical hormones and IL-6 levels following long-term magnesium supplementation in male students. *J Med Biochem* 2014; 33: 291–8.
26. King DE. Inflammation and elevation of C-reactive protein: does magnesium play a key role? *Magnes Res* 2009; 22: 57–9.
27. Libako P, Nowacki W, Rock E, Rayssiguier Y, Mazur A. Phagocyte priming by low magnesium status: input to the enhanced inflammatory and oxidative stress responses. *Magnes Res* 2010; 23: 1–4.
28. Sugimoto J, Romani AM, Valentin-Torres AM, Luciano AA, Ramirez Kitchen CM, Funderburg N, et al. Magnesium decreases inflammatory cytokine production: a novel innate immunomodulatory mechanism. *J Immunol* 2012; 188: 6338–46.
29. Uslu AU, Devenci K, Korkmaz S, Aydin B, Senel S, Sancakdar E, et al. Is neutrophil/lymphocyte ratio associated with subclinical inflammation and amyloidosis in patients

- with familial Mediterranean fever? *Biomed Res Int* 2013; 2013: 185317.
30. Tsubakihara T, Umeda T, Takahashi I, Matsuzaka M, Iwane K, Tanaka M, et al. Effects of soccer matches on neutrophil and lymphocyte functions in female university soccer players. *Luminescence* 2013; 28: 129–35.
31. Chamera T, Spieszny M, Klocek T, Kostrzewa-Nowak D, Nowak R, Lachowicz M, Buryta R, Ficek K, Eider J, Moska W, Ciężczyk P. Post-effort changes in activity of traditional diagnostic enzymatic markers in football players' blood. *J Med Biochem* 2015; 34: 179–90.
32. Wagner KH, Reichhold S, Neubauer O. Impact of endurance and ultraendurance exercise on DNA damage. *Ann N Y Acad Sci* 2011; 1229: 115–23.
33. Mucci P, Durand F, Lebel B, Bousquet J, Prefaut C. Basophils and exercise-induced hypoxemia in extreme athletes. *J Appl Physiol* (1985) 2001; 90: 989–96.
34. Hartwig A. Role of magnesium in genomic stability. *Mutat Res* 2001; 475: 113–21.

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