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Effect of astaxanthin supplementation on muscle damage and oxidative stress markers in elite young soccer players

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Aim. **The purpose of the current study was to examine the effect of Astaxanthin (Asx) supplementation on muscle enzymes as indirect markers of muscle damage, oxidative stress markers and antioxidant response in elite young soccer players.**

Methods. **Thirty-two male elite soccer players were randomly assigned in a double-blind fashion to Asx and placebo (P) group. After the 90 days of supplementation, the athletes performed a 2 hour acute exercise bout. Blood samples were obtained before and after 90 days of supplementation and after the exercise at the end of observational period for analysis of thiobarbituric acid-reacting substances (TBARS), advanced oxidation protein products (AOPP), superoxide anion ($O_2^{\cdot-}$), total antioxidative status (TAS), sulphydril groups (SH), superoxide-dismutase (SOD), serum creatine kinase (CK) and aspartate aminotransferase (AST).**

Results. **TBARS and AOPP levels did not change throughout the study. Regular training significantly increased $O_2^{\cdot-}$ levels (main training effect, P<0.01). $O_2^{\cdot-}$ concentrations increased after the soccer exercise (main exercise effect, P<0.01), but these changes reached statistical significance only in the P group (exercise x supplementation effect, P<0.05). TAS levels decreased significantly post- exercise only in P group (P<0.01). Both Asx and P groups experienced increase in total SH groups content (by 21% and 9%, respectively) and supplementation effect was marginally significant (P=0.08). Basal SOD activity significantly decreased both in P and in Asx group by the end of the study (main training effect, P<0.01). All participants showed a significant decrease in basal CK and AST activities after 90 days (main training effect, P<0.01 and P<0.001, respectively). CK and AST activities in serum significantly increased as result of soccer exercise (main exercise effect, P<0.001 and P<0.01, respectively). Postexercise CK and AST levels were significantly lower in Asx group compared to P group (P<0.05)**

Conclusion. **The results of the present study suggest that soccer training and soccer exercise are associated with excessive production of free radicals and oxidative stress, which might diminish antioxidant system efficiency. Supplementation with Asx could prevent exercise induced free radical production and depletion of non-enzymatic antioxidant defense in young soccer players**

Key words: Astaxanthine - Soccer - Oxidative stress.

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Aerobic exercise of sufficient intensity and duration can result in increased production of reactive oxygen species (ROS) in various tissues.[1] Prolonged exercise leads to the increased production of ROS by the mitochondrial electron transport chain through an increase in oxygen consumption.[2] Also, xanthine oxidase is activated via the ischemia–reperfusion process during exercise, resulting in the production of ROS.[3] The imbalance between enhanced ROS production and the ability of antioxidant systems to render them inactive, lead to cellular loss of redox homeostasis and to prone conditions of oxidative damage to cellular lipids, proteins and DNA.[4] Additionally, the emerging role of ROS in the delayed-onset muscle soreness and muscle injury has been recently reported.[5] ROS mediated sarcolemmal phospholipids peroxidation may play a role in the ethiology of exercise induced muscle damage.[6] The specific activity pattern of soccer training and match may favor additional pro-oxidant redox alterations.[7]

Methods to reduce free radical production and subsequent oxidative damage during and following physical exercise have been a priority of much research activity. Various antioxidants and their combinations were investigated. However, the use of antioxidants to attenuate exercise-induced muscle injury and oxidative stress has been met with mixed results. While some reports suggest a potential beneficial role of antioxidant supplementation in relation to exercise,[8, 9] others indicate no benefit.[10, 11] Discrepancies in findings may be due to the type, dosage, and timing of administration of the antioxidants, in addition to the exercise stress and the specific population being studied.

Astaxanthin (3, 3'-dihydroxy – β, β-carotene-4, 4'-dione; Asx) is a red carotenoid pigment, which occurs in certain marine animals and plants such as fish, shrimps, and algae.[12] Asx has unique chemical properties based on its molecular structure. The presence of the hydroxyl (OH) and keto (C=O) moieties on each ionone ring explains some of its unique features, namely, a higher antioxidant activity. It is able to scavenge radicals both at the surface and in the interior of phospholipids membrane.[13] Recent studies continue to evidence the multiple possibilities of Asx application in providing benefits to human health. Furthermore, Asx has also demonstrated cardioprotective,[14] neuroprotective [15] and anti-inflammatory properties (Figure 1).[16].

There has been little data reported on evaluation of Asx effect in sports field. There is evidence that Asx supplementation may increase muscle strength and endurance [17] and reduce muscle damage caused by physical activity.[18] Also, there several in vitro studies and in vivo animal models showing beneficial effect of Asx on reducing oxidative stress biomarkers.[12, 19]

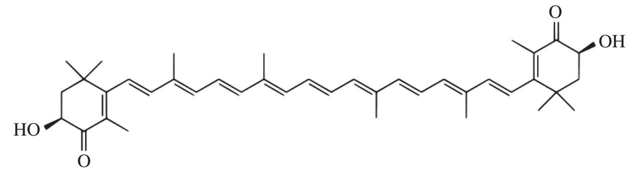

Figure 1.—Chemical structure of astaxanthin

Based on these findings, the purpose of the current study was to examine the effect of Asx supplementation on muscle enzymes as indirect markers of muscle damage, oxidative stress markers and antioxidant response after forced training. The influence of 90 days supplementation on the basal levels on antioxidant defenses, as well as on the basal oxidative stress markers and muscle enzymes was also analyzed.

Materials and methods

Subjects and protocol

Thirty-two male elite soccer players participated in this study. They are members of young selection of soccer club "Partizan", Belgrade, Serbia, champion among young selections of Serbian championship. All participants were in good health, had no ongoing or previous (during last year) injuries, not on any medication known to affect oxidative stress and were non-smokers. Subjects had 5 to 7 training sessions per week with an average weekly training of 10 to 15 hours and took participation in National championship during training season. They had different aspects of trainings, including strength, resistance, cardio, flexibility and proprioceptive training. The participation in the study did not have any effect on previously determined training and competition schedule. The physicians in outpatient clinic "Vita Maxima", Belgrade, Serbia evaluated physical performance of all participants during the study period and sport injury rates and incidence were recorded.

The study was undertaken in compliance with the Helsinki Decleration and approved by the ethical committee of Sports Medicine Association of Serbia. The soccer players and parents were informed about procedures, benefits and possible risks of participation in advance of the study. Verbal consent to participate in the study was witnessed and formally recorded. Prior to enrolment into the study, all subjects completed a submaximal oxygen consumption (VO_2 submax) test, body composition assessment, 4-day diet record and general health-screening questionnaire. The maximal oxygen consumption (VO_2max) was measured on a motor driven treadmill (Run race, Techno gym, Italy), using an indirect calorimetry system (Quark b2, Cosmed, Italy) during an incremental exercise test to volitional fatigue. The energy, macronutrient and micronutrient intakes were calculated using Cosmed FMed 2.0 software.

Subjects were randomly assigned in a double-blind fashion to one of two treatment groups. The Asx group (N.=18) was supplemented with 4 mg of Astaxanthin for 90 days. The astaxanthin used in this study was natural Asx derived from microalgae Haematococcus pluvialis supplied by Oriflame (Sweden). Dosage of 4 mg per day and during a study period of 90 days seems to be safe and hence, harmful side effects were not expected. The placebo (P) group (N.=14) was given capsules, identical in appearance and taste, but containing sacharose. Prior to entering the study and during the study, the participants were instructed to abstain from any antioxidant supplementation.

Determinations of basal antioxidant enzyme activity, oxidative stress markers and muscle damage markers were made before and after 90 days of supplementation. After this period, sportsman had two hour soccer exercise and samples were taken to determine same parameters before and after the exercise. During the exercise heart rate of each player was monitored using a pulsometer. As the relationship between the power output, the heart rate and the oxygen uptake is linear, we can indirectly evaluate the work done during training through the heart rate.[20] There are five metabolic zones, that are defined according to maximal oxygen uptake: Z1<70%, Z2: 70-80%, Z3: 80-90%, Z4: 90-100%, Z5: 100% or higher (Table I). By measuring heart rate during the training session, we calculated the average time each player work at each zone.[21]

The pre-exercise blood samples were obtained between 9-10 am after 10h overnight fast. After the first blood collection, before the exercise, subjects had a standardized breakfast (providing ~650 kcal: 16-17 g protein, 131-135 g carbohydrate, and 4-5 g fat; 190 IU vitamin A, 30 mg vitamin C, 0.5 mg vitamin E, 0.4mg copper, 2.1 mg manganese, 44.5 µg selenium and 2.1 mg zinc). The post-exercise blood samples were obtained 15 minutes after 2 hour soccer exercise.

TABLE I.—*Physical activity performed during the training.*

	Placebo	Astaxanthin
Z1 (%)	12.1±3.2	12.7±3.9
Z2 (%)	25.8±5.4	24.5±5.7
Z3 (%)	37.7±6.5	38.4±7.3
Z4 (%)	23.2±8.5	22.8±9.7
Z5 (%)	1.2±0.9	1.6±1.1

Z values represent persentige of time expended at each metabolic zone. Metabolic zones are defined according to maximal oxygen uptake: Z1<70%, Z2: 70-80%, Z3: 80-90%, Z4: 90-100%, Z5: 100% or higher.

Sample collection and analysis

Venous blood was collected into heparin evacuated tube (for plasma) and sample tube with serum separator gel (for serum) (Greiner Bio-one, Kremsmünster, Austria). Plasma and serum were separated by centrifugation and multiple aliquots of each sample were stored at -80°C until analysis. The following parameters were measured: thiobarbituric acid-reactive substances (TBARS), advanced oxidation protein products (AOPP), superoxide anion ($O_2^{\bullet-}$), total antioxidative status (TAS), sulphydryl groups (-SH) and superoxide- dismutase (SOD).

Plasma TBARS were measured using the TBARS assay employing the molar absorption coefficient of $1.56\times10^5 M^{-1} cm^{-1}$ at 535 nm, as previously described.[22] In our hands the intra-assay CV was 4.8% and the inter-assay was CV 7.2%; the reference value was 0.975±0.333 µmol/L. The AOPP were determined in the plasma using the method described by Witko-Sarsat *et al.*[23] This oxidative stress biomarker was detected in the plasma of chronic uremic patients. It was suggested that AOPP levels are a measure of highly oxidized proteins, especially albumin. Briefly, AOPP levels were measured by spectrophotometry at 340 nm in acidic condition and were calibrated with chloramine-T solutions that, in the presence of potassium iodide, absorb at 340 nm. AOPP concentrations were expressed in $\mu mol\times L^{-1}$ of chloramine-T equivalents. The intra-assay CV was 3.27% and the inter-assay CV was 6.5%; the reference value was 14.1±4.48 µmol/L). The rate of nitroblue tetrazolium reduction was used to measure the level of superoxide anion [24] (the intra-assay CV was 5.6% and the inter-assay CV was 9.5%; the reference value was 38.9±4.17 µmolNBT/min/L). TAS levels of sera were determined using a colorimetric, fully automated measurement method,[25] which was optimized and applied on ILab 300+ analyzer (Instrumentation Laboratory, Milan, Italy) in the laboratory of the Institute for Medical Biochemistry, Faculty of Pharmacy, Belgrade, Serbia. Potent free radical reactions were initiated with the production of hydroxyl radical (OH^-), which oxidize ABTS (2, 2'-Azinodi-[3 ethylbenzthiazoline sulphonate]) to a green cation radical ABTS+. Antioxidants, present in the sample, discolorate ABTS+ to a degree that is proportional to their concentrations. The results were expressed as vitamin E analogue which is used as a standard. The intra-assay CV was 1.94% and the inter-assay CV was 4.39%; the reference value was 0.859±0.017mmol/L. The concentration of sulphydryl groups in plasma was determined using 0.2 mmol/L5,5′-dithiobis (2- nitrobenzoic acid) (DTNB) reported by Ellmann [26] (the intra-assay CV was 2.86% and the inter-assay CV was 5.01%; the reference value was 0.523±0.062 mmol/L). Plasma superoxide-dismutase activity was measured according to a previously published method.[27] One unit of superoxide-dismutase activity is defined as the activity that inhibits the auto-oxidation of adrenalin by 50 %. The intra-assay CV was 6.3% and the inter-assay CV was 9.2%; the reference value was 120±21 U/L.

Serum creatine kinase (CK) and aspartate aminotransferase (AST) were assayed by routine enzymatic methods using an ILab 300+ analyzer and reagents purchased from Biosystems S.A. (Barcelona, Spain) and Bioanalytica (Belgrade, Serbia). Biochemical and hematological tests parameters were determined before administration and after 90 days of Asx supplementation.

Statistical analysis

Statistical analyses were performed using the Statgraphics 4.2 software (STSC, Inc. & Statistical Graphics Corporation 1985-1989), MS Excel and EduStat 2.01 (2005, Alpha Omnia, Belgrade, Serbia). All data were assessed for normality (Shapiro-Wilk test). The characteristics of the study population are presented in terms of mean values and standard deviations. When the distribution differed from a normal distribution, geometric means and 95% confidence intervals are given. Subjects' baseline physical characteristics and nutritional parameters were compared using independent-sample t-test. The effect of the Asx supplementation and regular training on the basal parameters was tested using 2 (Asx and placebo group) X 2 (before and after the supplementation) repeated measure analysis of variance ANOVA. The effect of antioxidant supplementation and soccer exercise was tested by 2 (Asx and placebo group) X 2 (before and after the exercise) repeated measure (ANOVA). Due to the fact that the distributions of AOPP, $O_2^{\bullet-}$, SOD, CK and AST were skewed, logarithmic transformation of the values was performed before statistical comparisons were made. Two-tailed p values are given throughout.

Results

Subject's physical characteristics are presented in Table II. There were no significant differences between treatment groups with respect to these characteristics at baseline.

The estimated daily energy and nurient intake of soccer players are listed in Table II. The Asx and P groups did not differ in the estimated average energetic and nutritional intake. The dietary analysis obtained from the 4-day food diary showed that the mean vitamin A and E intakes were below the dietary reference intake (DRI) recommendations for the Asx and P groups.[28, 29]

Regarding oxidative stress parameters, TBARS remained nearly unchanged in both groups, after observational period (Table III). We, also, did not detect any influence of 2 hours soccer exercise on TBARS production in Asx nor in P group. Basal AOPP levels did not change throughout the study. Soccer exercise at the end of supplementation period did not induced significant changes in AOPP levels in Asx or in P. As it could be seen in Table III, regular soccer training over the period of 90 days significantly, increased levels of $O_2^{\bullet-}$ in both groups of soccer players (main training effect, $P<0.01$). A significant exercise effect ($P<0.01$) and interaction effect among supplementation and exercise ($P<0.05$) on $O_2^{\bullet-}$ were found. $O_2^{\bullet-}$ concentrations increased significantly after the exercise in the P group, while there was no change in Asx group at the same time.

Basal TAS levels did not change along the study. However, 2X2 repeated measures ANOVA revealed significant exercise effect ($P<0.001$) and interaction effect among exercise and supplementation ($P<0.05$) on TAS levels. TAS levels decreased post- exercise in both groups, but this changes reached statistical significance only in P group ($P<0.01$).

At the beginning of the study, we noticed significant difference in SH groups content between Asx and P group ($P=0.05$). ANOVA repeated measures revealed significant training effect ($P<0.001$) on to-

TABLE II.—*Physical characteristics and nutrition analysis of the tested individuals before nutritional intervention.*

Characteristic	Asx	P
Age (year)	18.1±0.7	17.7±0.6
Weight (kg)	72.4±8.35	74.1±7.7
Height (cm)	177.6±6.9	180.7±6.4
Body mass index (kg/m2)	22.8±1.4	22.7±1.7
Fat (%)	10.5±2.5	10.6±3.6
VO2 (ml/min/kg)	55.1±5.3	52.1±3.5
Nutritional analysis (habitual dietary intake)		
Energy (kcal)	2932±657.8	3154±1107.1
Protein (g)	125±28.3	117±27.0
Fat (g)	101±29.3	96±25.4
Carbo hydrates (g)	366±102.7	383±102.4

TABLE III.—*Effect of the Asx supplementation on basal biomarkers of oxidative damage and antioxidative defence parameters.*

	Initial		Final	
	placebo	astaxanthin	placebo	astaxanthin
MDA (μmol/L)	1.11±0.14	1.08±0.18	1.07±0.18	1.05±0.25
AOPP (μmol/L)	22±14	28±20	27±15	28±13
$O_2^{\bullet -}$ (μmol/minL)	45±23	57±45	85±79a	99±94a
TAS (mmol vit.E equiv/L)	0531±0.139	0.551±0.139	0.585±0.113	0.538±0.108
SH groups (mmol/L)	0.557±0.088	0.493±0.060	0.595±0.059	0.598±0.060aa
SOD (U/L)	100±50	95±46	38±13a	47±30a

Values are expressed as mean±S.D. The difference in relation to before the supplementation was significant at P<0.01(aa) and at P<0.05 (a).

TABLE IV.—*Effect of the soccer training and Asx supplementation on biomarkers of oxidative damage and anti-oxidative defence parameters.*

	Before		After	
	Placebo	Astaxanthin	Placebo	Astaxanthin
MDA (μmol/L)	1.07±0.18	1.05±0.25	1.08±0.15	1.14±0.18
AOPP (μmol/L)	27±15	28±13	31±11	27±11
$O_2^{\bullet -}$ (μmol/minL)	85±79	99±94	175±137b	115±85
TAS (mmol vit.E equiv./L)	0.585±0.113	0.538±0.108	0.443±0.135bb#	463±0.162#
SH groups (mmol/L)	0.595±0.059	0.598±0.060	0.605±0.082	0.590±0.126
SOD (U/L)	38±13	47±30	45.58±20	52±26

Values are expressed as mean±S.D. The difference in relation to before the training was significant at P<0.01(bb) and at P<0.05 (b). The interaction effect (training x supplementation) was significant at P<0.05 (#).

tal SH groups content. Both Asx and P groups experienced increase in total SH groups content (by 21% and 9%, respectively) and supplementation effect was marginally significant (P=0.08). Basal SOD activity significantly decreased both in P and in the supplemented group at the end of the study (main training effect, P<0.01). The soccer exercise performed after 90 days of supplementation did not influenced SOD activity in the supplemented and in P group (Table IV).

We observed significant training effect on CK and AST levels during 90 days of study period (main training effect, P<0.01 and P<0.001, respectively). All participants showed a decrease in basal plasma CK and AST activities (CK decreased from 477 [341-667]U/L to 239 [158-363]U/L in Asx group and from 520 [265-1018]U/L to 388 [254-593]U/L, P group; AST decreased from 37 [29-48]U/L to 24 [20-28]U/L in Asx group and from 43[36-52]U/L to 29[24-34]in P group). There was no difference

TABLE V.—*Correlations between creatine kinase (CK) and aspartate aminotransferase (AST) levels during observational period*

	AST initial	
	placebo	astaxanthin
CK initial		
Spearman correlation	0.837**	0.599**
significance	0.000	0.003
n	18	14
	AST final	
	placebo	astaxanthin
CK final		
Speraman correlation	0.889**	0.573*
significance	0.000	0.026
n	18	14
	AST after the training	
	placebo	astaxanthin
CK after the training		
Spearman correlation	0.921**	0.580*
significance	0.000	0.015
n	18	14

* p<0,05; ** p<0,01

in basal CK and AST levels between the Asx and P group. CK and AST activities in serum significantly increased as result of soccer exercise (main exercise effect, P<0.001 and P<0.01, respectively). However, the effect of Asx supplementation on exercise induced changes in CK and AST levels was marginally significant (main supplementation effect, P=0.067 and P=0.062, respectively), with significantly lower post-exercise CK and AST levels in Asx group compared to P group (P<0.05).

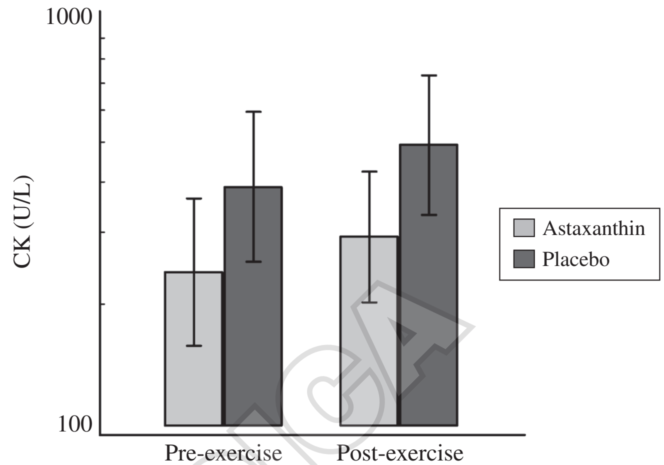

Figure 2.—Plasma creatine kinase (CK) activity (U/L) before and after exercise in placebo and astaxanthin group. Values are expressed as geometric mean values (95th confidence interval). The difference in relation to pre-exercise was significant at P<0.001. The difference in relation to Asx was signifficant at P<0.05.

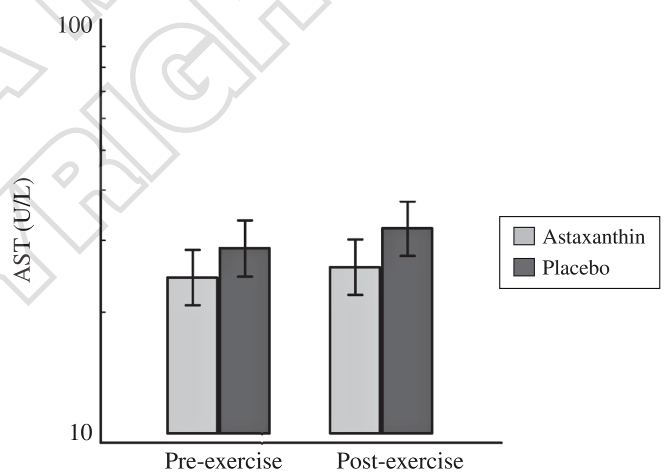

Figure 3.—Plasma aspartate aminotransferase (AST) activity (U/L) before and after exercise in placebo and astaxanthin group. creatine kinase (CK) activity (U/L) before and after exercise in placebo and astaxanthin group. Values are expressed as geometric mean values (95th confidence interval). The difference in relation to pre-exercise was significant at P<0.01. The difference in relation to Asx was signifficant at P<0.05.

Discussion

Excessive ROS production as a result of intense physical activity and subsequent oxidative stress certainly has the ability to result in physiological damage, but certain level of prooxidant production may actually serve as necessary stimulus for the upregulation of antioxidant defenses, thereby providing protection against future ROS attack[30]. On the other hand, some studies report decrease of antioxidant system efficiency and increase in the markers of oxidative stress in target tissues and blood in professional athletes subjected to high training and competitive load.[31, 32] It has been suggested that athletes under heavy training and competition are not able to maintain optimal tissue levels of antioxidant vitamins, even if the recommended daily allowances are consumed through their diets.[33]

The potential of dietary antioxidants as exogenous defense to minimize the extent of muscle injury or oxidative stress in response to exercise has received increasing attention in recent years. However, the results of works are not unequivocal and conclusive. This study aims to evaluate the effect of Asx supplementation in attenuating the oxidative stress and muscle damage induced by training protocol in field situation, while following their habitual dietary pattern and training and competition program.

Several studies have reported oxidative stress is markedly upregulated by a soccer game, observed though TBARS,[34] MDA levels [21] and serum hydroperoxide.[35] In addition, it was found that regular training and competition sessions resulted in increased basal oxidative stress as indicated by the increased MDA plasma levels in soccer players.[21, 36] In line with this, in the present investigation measured TBARS levels were above normal values, reflecting the high physical stress soccer players are exposed to during regular training and competition.

The beneficial effect of antioxidant vitamins on inhibition of peroxidation reactions has been an issue of many research papers. Although several studies indicate that antioxidant supplementation attenuates oxidative damage to lipids caused by exercise [8, 37] there are, likewise, published literature that suggests their ineffectiveness [10, 38] or that even report a prooxidative effect.[39] The results of our study did not show significant changes of basal TBARS levels or TBARS levels after the forced exercise. Also, we did not observed effect on this indicator of lipid peroxidation by Asx supplementation. Why dietary Asx did not reduce lipid peroxidation is unclear. Astaxanthin has been shown to be one of the most effective antioxidants against lipid peroxidation and oxidative stress in in vitro and in vivo systems.[12, 19] Astaxanthin is 100 times more active than α-tocopherol in protecting the rat mitochondria against Fe 2+ -catalyzed lipid peroxidation *in vivo* and *in vitro.*[40] TBARS is the most widely used biomarker of lipid peroxidation because it is inexpensive and easy to assay, there is some concern about its specificity and sensitivity. It has been demonstrated that antioxidant supplements significantly influenced lipid hydroperoxide and F2 -isoprostane levels in response to exercise, whereas they did not alter MDA, measured by TBARS.[41] This may account for the lack of a significant effect seen in this study.

AOPP is novel biomarker that may indicate long-term effects of oxidative stress such as after a high volume training period.[42] In the present study, AOPP levels were not different thought the season between our experimental groups, indicating that both groups had same level of oxidatively modified proteins. Also, we did not detect any changes in AOPP levels as result of soccer exercise in both groups. This is in agreement with other study on soccer players, which showed no increase in protein oxidation induced by soccer exercise training or match.[21, 36] Group of young soccer players analyzed in the present study had lower AOPP levels compared to the values observed in other studies,[42, 43] so it is possible that endogenous antioxidant defense mechanism was able to prevent significant changes in AOPP levels during observational period.

Continuous physical activity over the 90 days, seems to induce significant increase in $O_2^{\bullet-}$ production in soccer players. However, at the end of supplementation period, two groups responded differently to treatment, since only P group experienced significant increase in $O_2^{\bullet-}$ levels after the exercise, suggesting that Asx could, at least in part, decrease excessive production of $O_2^{\bullet-}$ during acute exercise bout in young soccer players. $O_2^{\bullet-}$ is the first ROS generated during different metabolic processes in cells and reacts rapidly with a variety of biological compounds such as sulphydryl, polyunsaturated fatty acids, and DNA[4]. By neutralizing $O_2^{\bullet-}$ Asx supplementation may be helpful in prevention oxidative damage of various biomolecules. In vitro studies regarding antioxidant properties of Asx also showed that Asx can be effective in reducing $O_2^{\bullet-}$ and H_2O_2 production.[15, 19]

Non-enzymatic antioxidant levels are modified as a result of aerobic exercise, but results are contradictory. Athletes often showed increased total antioxidant capacity in response to the oxidative stress imposed by intense physical activity.[44] Increased total antioxidant capacity is a consequence of vitamin E and C mobilization from their respective reserve in order to protect body against ROS[4] or significant augmented uric acid synthesis consecutive to an enhanced activation of xanthine oxidase.[44, 45] On the other hand, it appears that the antioxidant capacity may be temporarily reduced during and immediately postexercise, after which time levels typically increase above basal conditions during the recovery period.[46] In the present study, soccer exercise at the end of observational period induced significant decrease in TAS levels in P group, possibly indicating that plasma antioxidants are instantly utilized to eliminate increased levels of ROS. Soccer players followed diet low in vitamin A and vitamin E for at least 3 months. It is possible that endogenous antioxidants could not compensate low exogenous antioxidant intake for prolonged period of time.

On the contrary, there were no exercise-induced changes in TAS levels in the athletes who received Asx for 90 days. Despite Asx supplementation did not have ameliorated plasmatic antioxidant capacity at rest, in agreement with other works,[8, 45] it seemed to help to counterbalance the exercise oxidative insult. In line with this, supplementation with various antioxidants has been shown to significantly augment the exercise-induced TAS increase, whereas a no changes in TAS in resting conditions was observed.[47]

One of the indices of exercise-induced oxidant production is blood thiol oxidation. Cellular thiols are critically important in maintaining the cellular antioxidant defense network; in addition, thiols play a key role in regulating redox-sensitive signal transduction process[4]. Significant increase in SH groups' content with the number of years of training experience was observed in female volleyball players.[42] In accordance, total SH group's content increased as a result of continuous physical activity in young soccer players. These changes might be a part of a self-protecting mechanism against training-induced oxidative stress. The effect of Asx supplementation was marginally significant. Namely, at the beginning of our study, basal serum SH group levels appeared significantly lower in Asx group than in P group. After 3 months of Asx supplementation, this parameter was completely restored in Asx group. In line with this, Bonina et al. observed that dietary supplementation with red orange complex, rich in phenolic compounds (anthocyanins, flavanones, and hydroxycinnamic acids) and ascorbic acid increase serum level of SH groups' after 2 months.[1]

SOD functions in the cell as one of the primary enzymatic antioxidant defenses against superoxide radicals. Increases in SOD enzyme activity corresponds with enhanced resistance to oxidative stress.[4] The results of several studies suggest that levels of SOD activity in blood and muscle is increased in response to exercise interventions in a trained population.[4, 48] On the other hand, there are studies showing no changes or even decrease in SOD activity after endurance exercise.[49, 50] In our group of soccer players, there was significant decrease in SOD activity during study period. At the same time levels of $O_2^{\bullet-}$ increased in both groups. It is likely that continuous, exhaustive training sessions as well as the frequent competitive matches exposed participants to increased oxidative stress (observed through increased levels of $O_2^{\bullet-}$) that overwhelmed SOD activity. In general, modifications of antioxidant enzyme activities after exercise characterize either adaptation (an increase in the activity at first) or utilization (a decrease if oxidative stress is overwhelming).[4] These decreases had, hypothetically, been attributed to a modification of the catalytic centers and subsequent inactivation of enzymes due to a disturbed redox balance induced by augmented oxidative stress.[51] This is the first study investigating effect of Asx on endogenous enzymatic antioxidants in vivo. Although, beneficial effect of Asx on SOD activity was reported in several in vitro studies [12, 19] our concept of supplementation in young soccer players was not able to prevent decrease in SOD activity.

High degree of variability existed among soccer players with regard to oxidative stress biomarkers, indicating that some individuals were "responders", while others were "non-responders". Individual characteristics, dietary intake of antioxidants, position in the field might be possible factors causing these differences.

The efflux of muscle enzyme CK is considered to reflect a change in the normal membrane structure, induced by muscle damage, making it permeable to these molecules. In this sense, increased serum activities of CK is considered as indirect marker of muscle fiber injury.[30] AST activity is significantly increased immediately after muscular exertion, remaining at high levels for 24h. Therefore, in athletes, the implications of increased serum AST should be considered in combination with the activity of CK.[52]

In the present study, increased CK and AST activity in the young soccer players above normal values, at all time points, reflects the high physiological requirements of the soccer and also implies muscle damage. Over the period of 90 days of regular training and supplementation, there was a significant decrease in basal CK and AST levels. The change in CK and AST levels followed the same pattern in both Asx and P group. We believe that this reduction in basal muscle enzymes levels is part of adaptation process to intense physical activity over the 90 days of training regimen. The subjects' muscle tissues were strengthened by regular training, so the muscles become more resistant to exercise-related damage. Enzymatic adaptations consequent to long-term training were reported previously.[53]

The use of dietary antioxidants to reduce exercise-induced muscle injury has met with mixed success. It was shown that antioxidant vitamin supplementation does not appear to prevent exercise-induced muscle tissue damage.[54] On the other hand, dietary supplementation with antioxidant vitamins can decrease the exercise-induced increase in the rate of lipid peroxidation, which could help prevent muscle tissue damage.[8, 9]

Considering variety of events associated with signs and symptoms of muscle injury, it is unlikely that any specific antioxidant or combination of antioxidants would function to effectively eliminate muscle injury, but rather to reduce the degree of damage.[30] In the present study although serum CK and AST activities increased significantly postexercise in either group, significantly lower CK and AST levels were observed in the Asx group compared with the P group, suggesting that Asx supplementation can attenuate exercise induced muscle damage to some extent.

This is in accordance with the results of previously published studies regarding Asx supplementation and muscle injury. Aoi *et al.* showed that Asx can attenuate exercise-induced damage in mouse skeletal muscle and heart, including an associated neutrophil infiltration that induces further damage.[18] In experiment of Ikeuchi et al. the exercise induced increase in plasma CK activity was inhibited by treatment with astaxanthin.[17] The findings of lower CK and AST activity in soccer players supplemented with Asx might represent the ability of Asx to stabilize sarcolemma leading to less membrane disruption.

However, CK and AST activity should not be used alone as reliable index of muscle injury, considering that CK and AST do not correlate well with other markers of muscle injury such as muscle force, muscle performance or cytoskeletal disruption.[30]

Conclusions

The relationship between exercise and oxidative stress is no longer considered as a detrimental phenomenon, but rather stimulus for upregulation of antioxidant defences.[55] Although some studies documented inhibitory effect of antioxidant substances on adaptations to exercise, the potential role of antioxidant supplementation should be investigated.[56] The results of the present study suggest that soccer exercise and soccer training are associated with highly increased production of free radicals and oxidative stress (observed through $O_2^{\bullet -}$ and TBARS), which might diminish antioxidant system efficiency (observed through diminished SOD and TAS), as its components are used to quench the harmful radicals produced. Supplementation with Asx could prevent exercise induced free radical production and depletion of non enzymatic antioxidant defense in young soccer players. Observed reduction in basal muscle enzymes levels is part of adaptation process to intense physical activity over the 90 days of training regimen. However, Asx supplementation may be helpful in reducing serum peak of CK and AST after the forced soccer exercise.

Because of exercise and training modifications of antioxidant defense systems and low antioxidant dietary intakes in young soccer players, antioxidant supplementation in certain antioxidant nutrients such as Asx seems to be reasonable.

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