

## URINARY TOTAL ANTIOXIDANT CAPACITY IN SOCCER PLAYERS

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### Abstract

Both aerobic and anaerobic exercise contributes to oxidative stress by generation of free radicals. The human body is well equipped with both enzymatic and non-enzymatic antioxidant defence system. Soccer predominantly involves aerobic exercise with repeated bouts of anaerobic activities. The response of the different antioxidants to exercise might be sports-specific and hence the total antioxidant capacity (TAC) provides a better appraisal of the different antioxidant mechanisms of the body. TAC is the sum of the activities of antioxidants present in the material studied. The objective of the present study was to assess the urinary TAC (uTAC) in professional soccer players in different phases of the playing season and to compare the uTAC between professional, amateur and recreational soccer players. 21 professional, 20 amateur and 18 recreational players participated in the study. Results showed that the uTAC in the professional soccer players during pre-season (phase -1), early in-season (phase -2) and during the start of the end-season (phase -3) was (mean  $\pm$  SD)  $3.13 \pm 0.09$ ,  $2.73 \pm 0.37$  and  $2.99 \pm 0.41$  mmol·L<sup>-1</sup> respectively. The uTAC of the amateur and the recreational players during the start of end-season phase was  $2.89 \pm 0.44$  and  $1.77 \pm 0.66$  mmol·L<sup>-1</sup> respectively. Repeated Measures ANOVA revealed significant difference ( $p < 0.05$ ) in the uTAC between phase-1 and phase-2 while no significant difference was detected between the other phases in the professional soccer players. One-way ANOVA revealed significant difference ( $p < 0.05$ ) between the uTAC of the recreational players and the amateur and professional players while there was no significant difference ( $p > 0.05$ ) in the uTAC between amateur and professional players. In conclusion, the present study found that the uTAC in professional soccer players changes through the course of the competitive season especially at the start of the early in-season period. Further, this study also found that the uTAC in both amateur and professional was higher than in the recreational soccer players. Further research is required to determine the response of the specific antioxidants to soccer training and performance during the different phases of the season and at different levels of participation.

**Key words:** oxidative stress, antioxidant defence, urinary total antioxidant capacity, soccer players

### Introduction

Increased oxygen consumption by the body especially by the working muscles is a natural consequence of physical activity. Physical exercise leads to increased metabolic demand, which generates free radicals (Alessio, 1993; Sen, 1995). To prevent and counter the exercise-induced oxidative stress, the human body is well-equipped with an antioxidant defence system. In most tissues including skeletal muscles, pre-existing enzymatic and non-enzymatic antioxidant systems are available to buffer the concentration of the reactive oxygen species (Essig & Nosek, 1997). The antioxidant enzymes include catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidases (GPx) and the nonenzymatic system includes substances such as vitamins, reduced glutathione, uric acid and bilirubin (Sen, 1995). Soccer is characterised by a high-intensity intermittent exercise pattern where the majority of activities are aerobic in nature, with repeated bouts of anaerobic running throughout the game.

While aerobic exercise is suggested to be the predominant cause of increased oxidative stress (Goldfarb, 1993; Radak, Taylor, Ohno & Goto, 2001), acute bouts of anaerobic exercise also lead to increased oxidative stress (Bloomer & Goldfarb, 2004, Groussard et al., 2003). Moreover, both strenuous long-duration exercise and exhaustive sprint or high-intensity exercise can lead to oxidative stress (Marzatico, Pansarasa, Bertorelli, Somenzini & Della Valle, 1997; Radak et al., 2001). Therefore it can be hypothesized that the game demands of soccer would induce oxidative stress and elicit an antioxidant response in the players. Yet very few studies have assessed the phenomena of oxidative stress and antioxidant responses in the soccer players (Banfi et al., 2006; Brites et al., 1999; Cazzola, Russo-Volpe, Cervato & Cestaro, 2003). Apparently only two studies have estimated the total antioxidant response in the soccer players (Banfi et al., 2006; Brites et al., 1999), but with contradictory observations.

While one of the studies (Brites et al.) showed that the trained soccer players showed increased plasma total antioxidant capacity (TAC) compared to sedentary controls, the other study (Banfi et al.) reported no significant difference in either the magnitude of oxidative stress or the TAC between the soccer players and the sedentary controls. The response of various antioxidants to the exercise induced oxidative stimulus may not be a concomitant (Palazetti, Richard, Favier & Margaritis, 2003). Rather, there could be a sports-specific response of antioxidant activity to exercise (Dékány, Nemeskéri, Györe, Ékes & Pucso, 2002; Dékány et al., 2006). It is conceivable that there is a specific oxidative stress stimulus threshold for different antioxidants with respect to both the nature and intensity of the activity. Hence, while the estimation of selected antioxidants provides limited information about the response to oxidative stress, determination of the 'total antioxidant capacity' (TAC) might allow a better appraisal of the synergistic cooperation of the endogenous (in plasma and body fluids) and exogenous antioxidant systems (Goldfarb, 1999). Total antioxidant capacity (TAC) is a parameter characterizing the sum of the activities of the antioxidants present in the material studied (Ziobro & Bartosz, 2003). TAC has been mostly assessed in blood plasma and serum. Other body fluids are less frequently analyzed for TAC. Further, it is stated that the estimation of TAC level of body fluids other than blood plasma, especially of those available

non-invasively may provide additional information regarding the antioxidant status of the body (Ziobro & Bartosz, 2003). Among the different body fluids, urine is suitably used for the estimation of TAC (Kirschbaum, 2001; Koracevic, Koracevic, Djordevic, Andrejevic & Cosic, 2001; Tubaro, Ghiselli, Rapuzzi, Maiorino & Ursini, 1998). Moreover, the antioxidant capacity of human urine is found to be higher than blood plasma (Kirschbaum, 2001; Lissi, Salim-Hanna, Pascual & del Castillo, 1995; Ziobro & Bartosz, 2003). This is due the presence of greater quantities of uric acid in urine than in plasma. Uric acid is the major contributor of TAC in blood plasma (Tubaro et al., 1998; Ziobro & Bartosz, 2003) and also dominates the TAC of urine as it is present in higher concentrations in urine than in plasma (Kirschbaum, 2001, Lissi et al., 1995). The cited studies reporting TAC in soccer players are limited and are mainly cross-sectional in design. The TAC profile in elite soccer players at different times of the season is yet to be determined. Further, while the cited studies reported TAC in trained soccer players and compared them with sedentary controls, apparently the TAC of soccer players at different levels of participation is not yet documented. Moreover, while studies assessing TAC in soccer players have used plasma, the total urinary TAC in soccer players is yet to be reported. Therefore, the primary aim of the present study was to assess the urinary TAC in professional soccer players in different phases of the playing season.

Table 1 Physical characteristics of the participants (data collected during the start of end-season)

Participants	Age (yrs)	Stature (m)	Body mass (kg)
Professional (n=21)	17.7 ± 0.04	1.73 ± 0.04	67.2 ± 7.5
Amateur (n=20)	22.3 ± 1.6	1.74 ± 0.65	67.5 ± 6.6
Recreational (n=18)	22.5 ± 1.5	1.71 ± 0.52	65.2 ± 9.2

The training and match play profile of the participants is presented in Table 2.

Table 2 Training profile of the participants

Participants frequency (days per week)	Training duration (min per day)	Training training	Nature of frequency (per week)	Match playing
Professional	5	120-150	organised (Coach + Trainer)	1 (at least)
Amateur	3	90-120	organised (Coach + Trainer)	1
Recreational	irregular	non-uniform	self	not-regular

The secondary aim was to compare the urinary TAC between professional, amateur and recreational soccer players. The study hypothesized that the TAC of the professional soccer players would change through the season and would be associated with the level of soccer participation.

## Methods

### *Participants*

Twenty-one professional, 20 amateur and 18 recreational soccer players participated in the study. Goalkeepers were not included in the study. The physical characteristics of the participants are presented in Table 1. The professional players were the members of Singapore National under-18 team and also participated in the local professional soccer league. The amateur players were members of one of the top university teams in Singapore and participated in the national amateur championships. The recreational players were university students but with no regular training or regular match play schedule. The test procedures were explained verbally and provided in written form to the participants. The players below 18 years of age had parental consent (as they were below the age of legal consent) and all the participants gave written assent to participate in the study. The study was approved by the Institute's Ethical Committee for Human Subjects. The professional players were medically cleared for any organic diseases like diabetes, thyroid related diseases, hepatic and renal dysfunctions as a part of the mandatory requirements to be the member of the National team. The amateur and recreational players also had medical clearance for organic diseases during medical screening as a part of the university admission process.

### *Procedures*

Urine samples of the professional players were collected three times during the pre-season (January-February), early in-season (April-May) and start of the end-season (August-September) respectively. The urine samples of the amateur and the recreational players were collected along with the third phase of sample collection of the professional players. The time of the year was also the last phase of the season for the amateur players. While the recreational players did not have any structured season schedule, the time of the year is also considered as the last phase as their active soccer- playing months were from June to October. All the subjects were instructed to refrain from smoking, consuming pain-killer drugs (unless prescribed for therapeutic purpose), and taking supplements like ginseng, vitamin C and E, and alcohol

during the study. The professional players recorded the amount and type of food and fluids consumed for three days prior to the first sample collection during the study. Subjects were advised to be as accurate as possible with respect to recording the details of the diet. A simple diet record chart provided to the participants served as a guide for this purpose. Thereafter, three days prior to the urine sample collection during the other two phases in the season, the subjects were instructed to consume a similar diet as prior to the sample collection period in phase-1. The Assistant Coach of the team was assigned the responsibility of personally supervising the diet to be consumed and recorded by the players during the three-day recording period. Any questions or ambiguities with respect to diet and fluids were resolved individually and controlled through direct interviews and discussions. The amateur and the recreational players were also advised to consume similar food and fluids as recorded by the professional players for three days before the urine sample collection. Six research assistants were assigned the responsibility to procure the specifically prescribed food items from the stalls and provide it to the participants. This was possible since all the players belonging to the amateur and the recreational group were university students and were residing in the university halls of residence. None of the subjects were allowed to consume any other type of food during the three day period. The research assistants personally recorded the diet of all the participants. Furthermore, to doubly ensure that the players complied with the instruction for food and fluids, on arrival at the laboratory they were asked to recall the diet consumed for the three days while the researcher matched it from the diet record sheet. The urine samples were collected during mid-week or at least 4 days after a match. The post-match day was a rest day for the entire team. Thereafter, all the players had a similar volume and intensity of training during the three days prior to the sample collection. This ensured similar physiological and metabolic status of the subjects. The urine samples were collected in the morning prior to any physical activity. The players were also advised not to consume caffeinated drinks and heavy meals for at least 3 hours prior to the urine sample collection. There were no restrictions on consumption of water. To ensure adequate hydration, the specific gravity of the urine samples were tested using a reagent strip (Urine 4S, Lot 06999, Catalogue no. 102008, Smartest Diagnostics, Yavne, Israel). Any urine sample with specific gravity of greater than 1.010 (Casa, Armstrong, Montain, Rich & Stone, 2000) was not accepted for analysis.

The urine samples were collected in clean plastic test tubes and immediately stored on ice. The TAC assays were performed within three hours of the sample collection.

### Urinary total antioxidant capacity

The urinary TAC was estimated using a commercially available antioxidant assay kit (709001, Cayman Chemical, MI, USA). The various endogenous and exogenous antioxidants in the body are either aqueous or lipid soluble. In the assay protocol using this kit, these two types of antioxidants are not separated. Thus, the combined antioxidant activities of all its constituents like vitamins, proteins, lipids, glutathione and uric acid are assessed. The TAC of the urine was determined by the method based on the absorbance of ABTS<sup>•+</sup> radical cation (2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) as previously described by Miller, Rice-Evans, Davies, Gopinathan & Milner (1993). The assay was based on the ability of antioxidants in the urine sample to inhibit the oxidation of 2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS<sup>•+</sup>) to ABTS<sup>•+</sup> by metmyoglobin. The amount of ABTS<sup>•+</sup> produced was monitored by reading the absorbance at 750 nm using a plate reader (SPECTRAMax 384 PLUS, Molecular Devices, CA, USA). Under the reactive conditions, the antioxidants in the sample caused suppression of the absorbance at 750 nm to a degree, which is proportional to their concentration (Miller et al., 1993). The capacity of the antioxidants to prevent ABTS<sup>•+</sup> oxidation was compared with that of Trolox, a water-soluble tocopherol analogue, and was quantified as millimolar Trolox equivalents. The antioxidant concentration of the samples was estimated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the following equation:

$$\text{Antioxidant (mmol)} = \frac{\text{Sample average absorbance} - \text{y-intercept}}{\text{slope}} \times \text{dilution}$$

The suitability of using Trolox as a standard (Koracevic et al., 2001) and ABTS as an assay system (Kirschbaum, 2001) to determine the TAC of urine has been reported in previous studies. To obtain reproducible results, antioxidant levels of the sample should fall within the standard curve. Urine was diluted 1:10 with the assay buffer before assaying as pilot studies in our laboratory showed that the results of the duplicate sample analysis were more stable with 1:10 dilution and were inconsistent with higher dilutions.

All reagents except samples were equilibrated to room temperature before beginning the assay. Compatible software (Softmax<sup>®</sup> Pro, Version 4.0 for Macintosh and Windows) was used for data analysis.

### Statistical analysis

All the results were expressed as mean  $\pm$  SD. One-way Repeated Measures Analysis of Variance (RM-ANOVA) was used to analyse the urinary TAC in the professional soccer players at different times of the playing season. Greenhouse-Geisser correction was used in the event of violation of sphericity. A Bonferroni post-hoc analysis was performed in the event of a significant main difference. The TAC between the different groups of players was analysed using one-way ANOVA with Tukey's post-hoc test where appropriate. The level of significance was accepted at  $p < 0.05$ . Statistical Package for Social Sciences (SPSS) version 14.0 was used.

### Results

The urinary TAC of the professional soccer players during the three phases of the season was found to be (mean  $\pm$  SD)  $3.13 \pm 0.09$ ,  $2.73 \pm 0.37$  and  $2.99 \pm 0.41$  mmol·L<sup>-1</sup> respectively. The urinary TAC of the amateur and the recreational players was  $2.89 \pm 0.44$  and  $1.77 \pm 0.66$  mmol·L<sup>-1</sup> respectively. Significant main effect was detected in RM-ANOVA analysis ( $F(1.55, 32.63) = 8.12$ ;  $p < 0.05$ ) of the urinary TAC in the professional soccer players during the three phases of the season. Pairwise post-hoc comparisons revealed significant difference ( $p < 0.05$ ) between the urinary TAC during the pre-season and the early in-season period (Figure 1). However, no significant differences were detected in the uTAC between the pre-season and the end-season or the early in-season and the end-season period.

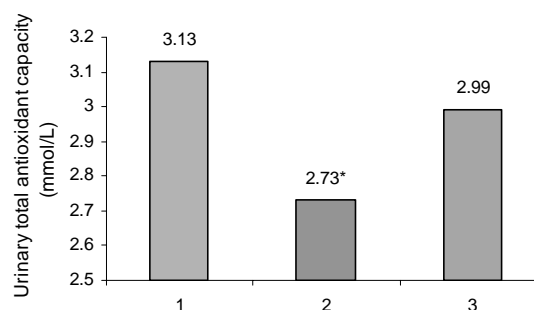


Figure 1. Urinary TAC in professional soccer players.

Note: 1- pre-season; 2- early in-season; 3- start of end-season; mmol/L – millimoles per litre; \* significantly different from the pre-season measure at  $p < 0.05$

This study also estimated uTAC in the amateur and recreational soccer players simultaneously during the third phase of sample collection in the professional soccer players. The one-way ANOVA revealed a significant main effect ( $F(2, 41) = 24.26, p < 0.05$ ). Tukey's post-hoc test showed that the urinary TAC of the recreational group was significantly different ( $p < 0.05$ ) from the professional and the amateur group (Figure 2) while the TAC in the amateur and the professional group was not significantly different ( $p > 0.05$ ).

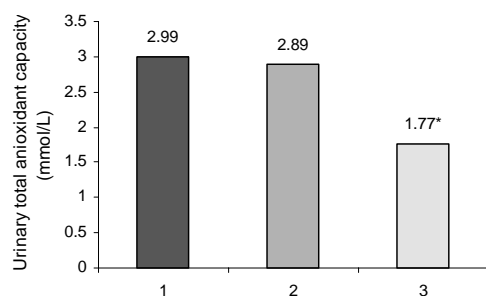


Figure 2. Urinary TAC in professional, amateur and recreational soccer players.

Note: 1- professional soccer players; 2- amateur soccer players; 3- recreational soccer players; mmol/L – millimoles per litre; \* significantly different from the professional and amateur soccer players at  $p < 0.05$

## Discussion and conclusion

The primary aim of the present study was to assess the uTAC in professional soccer players at different times during the playing season and the secondary aim was to compare the uTAC between professional, amateur and recreational soccer players. Apparently, this is the first study to report the uTAC in professional soccer players during different phases of the playing season and to compare the uTAC between soccer players at different levels of participation. The present study showed that the level of the uTAC in the professional soccer players changed during the playing season and a significant difference in the uTAC was also detected between the pre-season and the early in-season values. The results also showed that while the uTAC in the recreational soccer players was significantly lower than in amateur and professional soccer players, there was no significant difference in the uTAC between the amateur and the professional soccer players. Based on the rationale that both aerobic and anaerobic activity contribute to oxidative stress and elicit an antioxidant response in soccer, it was hypothesised that the uTAC would change during the different phases of the season in the professional soccer players.

Furthermore, the uTAC levels would be different in soccer players depending on the levels of participation. The findings of the present study favourably supported this hypothesis. The previous studies estimating TAC in soccer players are limited by being cross-sectional (Banfi et al., 2006, Brites et al., 1999). Furthermore, the cited studies were contradictory in their observations in plasma TAC between soccer players and sedentary controls. The results of the present study showed a significant decrease during the early in-season period compared to the pre-season and end-season respectively. This finding supported the hypothesis that the uTAC of professional soccer players changes through the season. Similar results have been reported in a study on professional rugby players, in whom the activity pattern can be reasonably considered to be similar to that in soccer (Finaud et al., 2006). The authors reported that there was a decrease in the plasma TAC during the beginning of the competitive period of the season and thereafter an increase with the progress of the competitive season.

It is conceivable that as the competition season commences, there is an increase in the volume as well as in the intensity of exercise with respect to both training and match play. The nature of training becomes that of high-intensity aerobic type and also with a greater anaerobic load. Moreover, owing to the competition demands, it is also highly possible that the recovery during this period would be insufficient in the players. Such an increase in exercise load would cause a significant increase in the oxidative stress due to increased generation of free radicals due to both a higher aerobic load (Alessio, 1988, 1993); and a higher anaerobic load (Bloomer & Goldfarb, 2004; Groussard et al., 2003). Theoretically, this should have led to an increased antioxidant response. On the contrary, there was a significant decrease in the uTAC in the professional soccer players from the pre-season phase to the early in-season phase of the soccer season. This finding can be explained based on the understanding that an excessive production of free radicals severely hampers the antioxidant defences and causes changes in the cellular homeostasis mechanisms (Child, Wilkinson, Fallowfield & Donnelly, 1998; Marzatico et al., 1997). High performance levels associated high training demands compounded with insufficient recovery can damage antioxidant proteins, deplete cellular scavenger stores and may provoke a transitory or prolonged lack of physiological and/or biochemical adaptations (Palazetti et al., 2003; Fry, Morton & Keast, 1991).

Moreover, it has also been reported that the plasma TAC significantly decreased in overloaded triathletes suggesting that overload training also causes consumption of non-enzymatic antioxidants (Palazetti et al., 2003). The results of this study showed that there was an increase in the uTAC, although not statistically significant, with the progress of the soccer season. Regular training is known to upregulate the antioxidant defense mechanisms especially the muscle antioxidant enzyme activity, providing additional protection during the times of intense physical stress (Powers, Ji & Leeuwenburgh, 1999) and this is the case for both aerobic as well as anaerobic exercise (Radak et al., 2001). Previous studies on soccer players (Banfi et al., 2006, Brites et al., 1999) have compared the plasma TAC in soccer players with that in sedentary controls, but apparently no study has compared the urinary TAC between soccer players at different levels of participation. The present study showed that the players at the professional and even at the amateur levels had higher TAC than the recreational players. The result confirms that the antioxidant response parallels the increase in oxidative stress (Radak et al., 2001). The present study showed that the magnitude of oxidative stress and the antioxidant response increase with an increase in the training and competition load in soccer. The urinary TAC is reported to be higher than the plasma TAC (Kirschbaum, 2001; Lissi et al., 1995; Ziobro & Bartosz, 2003). The findings of the present study support this observation. Previous studies have reported plasma TAC (mean) of 1.33 mmol·L<sup>-1</sup> in trained soccer players (Banfi et al., 2006), 1.80-1.87 (range) in professional rugby players (Finaud et al., 2006) and 1.45-1.51 (range) in well-trained triathletes (Palazetti et al., 2003). The urinary TAC of the amateur soccer players in the present study was (mean ± SD) 2.89 ± 0.44

mmol·L<sup>-1</sup> and that in the professional players was (range) 2.73-3.13 mmol·L<sup>-1</sup> respectively thereby supporting the theory of urinary TAC being higher than the plasma TAC.

This deduction although reasonable, would have been further substantiated by estimations of plasma TAC of the participants in the present study. This can be considered as a limitation of the present study. The findings of the present study constitute the ground for future studies on oxidative stress and antioxidant response in soccer players. Though the present study determined the uTAC in the soccer players, further studies are necessary to determine if the activity demands in soccer induces a specific antioxidant enzyme response. Further studies are also required to determine the effect of specific physiological parameters like aerobic capacity, repeated sprint ability and the metabolic load of training on the antioxidant response of players at different levels of participation. Furthermore, to obtain greater insights on the implications of antioxidant response in soccer, studies determining the relationship between antioxidant response and specific performance qualities in soccer would be of practical significance. In conclusion, the present study found that the uTAC in professional soccer players changes through the course of the competitive season. Increase in the training and competition load at the start of the in-season period can lead to an overwhelming period of oxidative stress causing a lack or a lag in the antioxidant adaptations. This study hence emphasizes the possible significance of determining the oxidative stress and the antioxidant response as a part of the biological follow-up in professional soccer players. Further, this study also showed that the uTAC in trained soccer players, both amateur and professional was higher than in the recreational soccer players.

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## URINARNI TOTALNI ANTI-OKSIDATIVNI KAPACITET NOGOMETAŠA

### **Sažetak**

Obje vrste vježbanja, i aerobno i anaerobno, doprinose oksidacijskom stresu stvaranjem slobodnih radikala. Ljudski organizam je dobro opremljen s oba antioksidativna obrambena sustava, enzimskim i neenzimskim. Nogomet predominantno uključuje aerobno vježbanje s ponovljenim djelovanjima anaerobnih aktivnosti. Reakcija različitih antioksidanata na vježbanje može biti specifična po sportu, stoga totalni antioksidativni kapacitet (TAC) omogućava bolju procjenu različitih antioksidativnih mehanizama tijela. TAC je suma aktivnosti antioksidanata u studiranom materijalu. Cilj ovog istraživanja je bio procjena urinarnog uTAC-a kod profesionalnih nogometaša u različitim fazama natjecateljske sezone kao i usporedba uTAC-a između profesionalnih, amaterskih i rekreacijskih igrača nogometa. Ukupno 21 profesionalac, 20 amatera i 18 rekreativaca je sudjelovalo u istraživanju. Rezultati su pokazali da je uTAC kod profesionalnih nogometaša u pred-sezoni (faza-1), rano u sezoni (faza-2) i na početku završnog dijela sezone bio: (aritm.sr.  $\pm$  st.dev.)  $3.13 \pm 0.09$ ,  $2.73 \pm 0.37$  i  $2.99 \pm 0.41$  mmol·L<sup>-1</sup>, respektivno. Vrijednosti uTAC-a kod amatera i rekreativaca na početku sezone je bio  $2.89 \pm 0.44$  i  $1.77 \pm 0.66$  mmol·L<sup>-1</sup>, respektivno. Ponovljena mjerenja primjenom Analize varijance su otkrila značajnu razliku ( $p < 0.05$ ) uTAC-a između faze-1 i faze-2 dok nisu pronađene značajne razlike između ostalih faza kod profesionalaca. Analiza varijance otkrila je značajnu razliku ( $p < 0.05$ ) uTAC-a između rekreativaca, amaterskih igrača i profesionalaca pri čemu nije bilo značajne razlike ( $p > 0.05$ ) između amaterskih i profesionalnih igrača. Zaključno, prikazana studija pronašla je da se uTAC kod profesionalnih nogometaša mijenja za vrijeme trajanja natjecateljske sezone posebno na početku faze rane sezone. Nadalje, ova studija je također pronašla da je uTAC i kod amaterskih i profesionalnih nogometaša viši nego kod rekreativnih igrača nogometa. Buduća istraživanja trebaju odrediti reakciju specifičnih antioksidanata u nogometnom treningu i utakmici za vrijeme različitih faza sezone i uz različitu razinu sudjelovanja pojedinaca.

**Ključne riječi:** oksidativni stres, antioksidativna obrana, totani anti-oksidativni kapacitet, nogometaši

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