

Relationships between Metabolic Profiles, Redox Homeostasis and Adiponectin in Young Athletes: Soccer vs. Basketball

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Abstract

Background: Skeletal muscle is the main contributor to reactive oxygen species during physical exercise. In elite athletes, the training load conditions the relationship between redox homeostasis and the metabolic profile of the muscle with the production of peripheral signals. The goal of this study was to investigate the relationship between redox homeostasis and peripheral signals in two sports characterized by a different workload, football and basketball.

Methods: A cohort of 38 male professional players (20 football and 18 basketball) were enrolled in the study. The antioxidant capacity and the levels of reactive oxygen metabolites on plasma were determined by using the d-ROMs test and the BAP Test. A GC-MS-based metabolic profiling approach was used.

Results: Soccer players had higher levels of oxidative species (dROM+31.25%), with lower antioxidant capacity (BAP value -16.8%), and plasma adiponectin level (-48%) than basketball players ($P < 0.01$ for all). Metabolic profiling of compounds differentially expressed between the two groups of athletes indicates the involvement of urea cycle and tyrosine metabolism.

Conclusion: Present results indicate that soccer athletes are subject to higher oxidative stress in comparison with basketball elite players. This pilot study indicates the need to evaluate whether these peripheral signals can be a marker of the athlete's condition; a marker of the achievement of overreaching; or a guide to developing more effective training techniques.

Keywords: Metabolic profile; Redox homeostasis; Young athletes; Inflammation

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Introduction

The measurement and monitoring of recovery and fatigue during training periods contribute to the correct method to improve sports performance. In elite athletes a decrease in short-term performance will lead, after the recovery phase, to improve training and sport activity. However, when this balance between intensive training and recovery is not respected, athletes can experience a drop in sports efficiency that is often no recoverable. This can lead to Overreaching/Overtraining Syndrome (OTS) [1]. The signals of OTS are hormonal, oxidative and metabolic stress. As reported skeletal muscle is the main contributors to the exercise-induced Reactive Oxygen Species (ROS) production that play an important role in signalling [2]. Since ROS up regulate the expression of antioxidant enzyme leading to a stimulation of the antioxidant defence system, they exhibit physiological stimulus

for muscle regeneration. However, prolonged intense physical activity could increase muscle damage and lead to a modification on redox homeostasis parameters and to a cascade of metabolic signal in the body that, in professional athletes, reduce physical performance [3]. In conclusion, inadequate training programs that involve repeated bouts of exercise could be responsible to the decline on performance associated to an increase in Oxidative Stress (OS) and inflammation. Chronic training stress is responsible of perturbations in several metabolic hormones related to inflammation. Adiponectin is a signalling hormone with an anti-inflammatory effect and its plasma level increases in well-trained athletes. This suggests that training could change the adiponectin response and further that its decrease could be a

sign of overreaching/overtraining [1]. It is interesting to evaluate the effects of specific training protocol on several peripheral signals such as leptin, ghrelin and adiponectin plasma levels since they are related with health status and glucose and lipids metabolism and because acute and chronic exercises affect body composition, carbohydrate and lipid metabolism. Metabolomics technologies are useful to understand the athlete's conditions and show potential applications in sport science to identify metabolic biomarkers to understand the physical performance or to predict talent identification [4]. The aim of this study is to determine the metabolic profile and redox homeostasis in elite athletes exposed to high-training loads who practice at a competitive level, soccer and basketball. In detail, the primary objective of this study was to analyse the correlation between adiponectin and redox homeostasis in athletes practising two different sports and to provide an overview of their prevalence comparing the two sports. Moreover, this study aimed to investigate the relationship among peripheral signals and redox homeostasis biomarker in soccer and basketball players during a match season. We choose soccer and basketball for their characteristics. They require specific skills such as the ability to move at high speed or change direction quickly and both athletes tend to possess high strength, power and agility. The metabolism used in these sports is very similar and is predominantly anaerobic/aerobic alternate although in basketball, the aerobic demand is less than soccer. Therefore, these peripheral signals may be used: a) to reveal the condition of the athlete; b) as markers of overreaching/overtraining and c) to identify the most effective training techniques.

Materials and Methods

Materials

Unless specified all reagents were obtained from Sigma (St. Louis, MO) except PVDF membrane (Millipore, Bedford, MA).

Participants

A cohort of 20 male professional soccer players and 18 male professional basketball players from different teams were involved in this study. All participants consumed a diet without any nutritional supplements and were recruited from different regional elite teams. Each athlete follows a specific training program proposed by the coach and adapted to specific sports competitions. The recruited players followed a training routine of five days per week, with sessions lasting 2 hrs per day. The training protocol involved technical and aerobic exercise. Team coaches tracked day-to-day training data for each player and for each training session over the entire season. Therefore, workouts, schedules, calendar as well as methods of training were individualized per teams and athletes, so the only criteria about training programs we used to select the participants from a larger cohort was the amount of training times per week and the level set by regional competitions they participate. To assess their adherence to the training plans, the subjects completed the physical activity questionnaires. To date, no method has proved effective in quantifying high intensity training and in the present study the athletic trainers used the Rating of Perceived Exertion

(RPE) system to monitor Training Load (TL) to know the individual responses of athletes in different training sessions [5]. All the experiments are conducted with male athletes who voluntarily choose to participate following explanation of all experimental procedures. A medical history and physical activity questionnaire was completed by the subjects in order to determine the eligibility. No participants used antioxidant supplements. Athletes were selected because of non-smoking status, age, stable body weight, and maintenance of regular exercise patterns. After initial screening, all participants received a complete explanation of the purpose, risks and procedures of the study. A written informed consent was provided prior to enrolment in the study, it was conducted according to policy statement set forth in the Declaration of Helsinki, and all the experiments were conducted according to established ethical guidelines. The study was approved by the local Ethics Committee of the University of Florence, Italy (AM_G sport 15840/CAM_BIO). All measurements are performed in resting conditions and by the same operator. During the study period, the trained subjects had their optimal body composition (i.e., lowest fat mass and highest fat-free mass). Weight is measured to the nearest 0.1 kg and height (H) to the nearest 0.5 cm. Body mass index (BMI) was calculated from the ratio of body weight (kg) to body height (m²). A fasting capillary blood sample is taken using a heparinized microvette from each volunteer after obtaining written and informed consent. The capillary collection was preferred to the venous one because of its reduced invasiveness, simple execution and lower cost; moreover small volumes of samples were sufficient for the experiments carried out. The biological samples are collected far from a competition in morning and before the training session.

Plasma oxidative stress measurements

The antioxidant capacity and the levels of reactive oxygen metabolites on plasma were determined by using the d-ROMs test (diacron reactive oxygen metabolite) and the BAP Test (Biological Antioxidant Potential) used to determine the efficiency to balance the production of free radicals in terms of iron-reducing activity. The biomarkers dROM and BAP were selected based on their long-term stability. All serum analyses were performed using a free radical analyzer system (Free Carpe Diem, Diacron International s.r.l) that included a spectrophotometric device reader and a thermostatically regulated mini-centrifuge, and the measurement kits were optimized to the FREE Carpe Diem System, according to the manufacturer's instructions. The dROM test reflects the amount of organic hydro peroxides that is related to the free radicals from which they are formed. When the samples are dissolved in an acidic buffer, the proteins in the acidic medium liberated the metal ions (Fe) and the hydro peroxides react with them and are converted to alkoxy and peroxy radicals. These newly formed radicals oxidize an additive aromatic amine (N, N diethylparaphenylen-diamine) and cause formation of a relatively stable colored cation radical that is spectrophotometrically detectable at 505 nm. The results are expressed in arbitrary units (U. Carr), one unit of which corresponds to 0.8 mg/L of hydrogen peroxide. The BAP test provides an estimate of the global antioxidant capacity of sample, measured as its reducing

potential against ferric ions. The intensity of the discoloration resulting by mixing a ferric chloride solution with a thiocyanate derivative solution is spectrophotometrically detectable at 505 nm and is proportional to the ability of plasma or serum to reduce ferric ions. The results are expressed in $\mu\text{mol/L}$ of the reduced ferric ions [6].

Adiponectin western blot analysis

Plasma samples were clarified by centrifugation and total protein contents were obtained using Bradford assay. An equal amount of each sample (10 μg of total proteins) was added to 4 \times Laemmli buffer (0.5 M TrisHCl pH 6.8, 10% SDS, 20% glycerol, β -mercaptoethanol, 0.1% bromophenol blue) and boiled for 10 min. Samples were separated on 12% SDS/PAGE and transferred onto PVDF membrane using Trans-Blot Turbo Transfer System (BIO-RAD). PVDF probed with primary antibody (Acrp30 Santa Cruz) diluted 1:1000 in 2% milk and incubated overnight at 4°C. After incubation with horseradish peroxidase (HRP)-conjugated antimouse IgG (1:10000) (Santa Cruz Laboratories), immune complexes were detected with the Enhanced Chemiluminescence (ECL) detection system (GE Healthcare) and by Amersham Imager 600 (GE Healthcare). For quantification, the blot was subjected to densitometry analysis using ImageJ program. The intensity of the immunostained bands was normalized with the total protein intensities measured by Coomassie brilliant blue R-250 from the same PVDF membrane blot as previously reported [6].

Gas chromatography–MS (GC-MS) and sample treatment

100 μl of plasma samples were subjected to metabolite analysis by GC-MS as reported by Luti et al. [6]. The injection volume was 1 μl and a split ratio of 1:10 was used. The Mass Hunter data processing tool (Agilent) was used to obtain a global metabolic profiling using the Fihen Metabolomics RTL library (Agilent G1676AA).

Statistical analysis

Data are presented as means \pm Standard Deviation (SD) from at least three experiments. Statistical analysis was performed by two-tailed t-Test using Graph pad Prism 8. Significance was defined as $P < 0.05$. For western blot quantification, the blot was subjected to densitometric analysis using ImageJ program. For correlation analysis of adiponectin level with each parameter measured, non-parametric correlation Spearman test was performed using Graph pad Prism 8. A 90% confidence interval as range of values was used and $P < 0.1$ was considered as significant.

Results

Participant's characteristics

Since the aim of the study is not to evaluate how a specific training programs effects athletes performance the measurement to determine metabolic profile of athletes were performed during "regular season", both for soccer and basketball. Descriptive characteristics of the participants are presented in **Table 1**. Mean age was 20.78 ± 1.53 years for all the athletes; mean weight

was 81.5 ± 9.3 kg and height was 186.15 ± 5.4 cm for basketball players; for soccer players mean weight was 72.7 ± 8.5 kg and height was 179.15 ± 6.04 cm. Basketball players were significantly heavier (p -value=0,0144) and taller (p -value=0.0028) than soccer players. BMI was calculated and no significant differences were found between groups (22.46 ± 1.73 for all); enrolled athletes did not have metabolic disorders and did not take specific drugs as reported in methods.

Characteristics	Mean (SD)			
	All	Basketball players	Soccer players	P
Age (year)	20.78 \pm 1.53	21 \pm 2.08	20.65 \pm 1.1	0.5364
Weight (kg)	79.9 \pm 9.8	81.5 \pm 9.3	72.7 \pm 8.5	0.0144*
Height (cm)	181.6 \pm 6.7	186.15 \pm 5.4	179.15 \pm 6.04	0.0028*
BMI (kg/m ²)	22.46 \pm 1.73	22.83 \pm 2.01	22.13 \pm 1.36	0.2305

Note: *Statistically significant difference.

Table 1: Participants' characteristics.

Plasma oxidative stress measurements

We measured the antioxidant capacity (BAP) and the levels of oxidative species (dROM) from all the athlete's groups and the results are reported in **Figure 1**. We noticed that comparing BAP and dROM (**Figures 1A and 1B**) in plasma significant differences are shown between the two groups. In detail analyzing the antioxidant capacity (BAP value), a significant reduction (16.8%; $P < 0.0001$) in soccer players is observed; as shown in **Figure 1A** the BAP mean value was 1901.58 ± 285 $\mu\text{mol/L}$ in comparison with basketball players 2285.7 ± 223 $\mu\text{mol/L}$. Moreover, the same athletes show an increase of about 31.25% ($P < 0.0001$) of the levels of oxidative species (dROM), the mean value was 338.47 ± 46 UCarr for soccer and 232.68 ± 40 UCarr for basketball players.

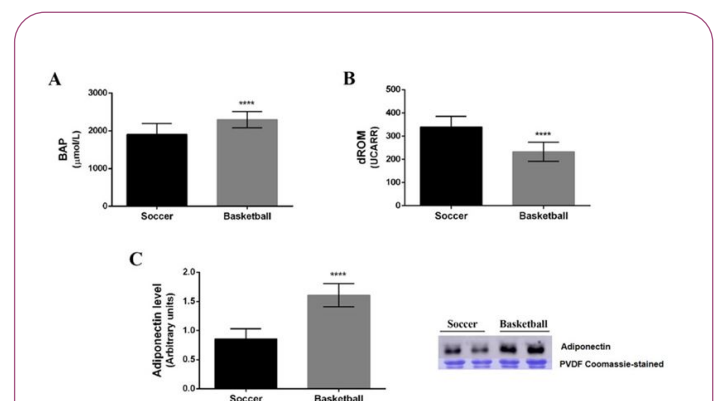


Figure 1: Plasma oxidative stress and Adiponectin determination. A) The antioxidant capacity was evaluated using the the BAP Test (Biological Antioxidant Potential), B) the levels of reactive oxygen metabolites using the d-ROM test (diacron Reactive Oxygen Metabolite) by a free radical analyzer system (Free Carpe Diem, Diacron International s.r.l), and C) Adiponectin level was assessed in 5 μg of plasma athletes treated with reducing agent by western blot with adiponectin antibody normalized on protein content, have been. Data represent mean \pm SEM, $n=20$ soccer and 18 basketball. A representative immunoblot is shown together with the corresponding Coomassie-stained PVDF membrane. All the measurements ($n=20$ soccer and 18 basketball) were performed in triplicate and are reported in the histograms as mean \pm SD. The statistical analysis was carried out by two-tailed t-Test using Graph pad Prism 8 (**** $P < 0.0001$).

Plasma adiponectin determination

The plasma adiponectin level was determined by western blot in both groups of athletes and the results are reported in **Figure 1C**. We observed minor signal intensity in soccer players in comparison with basketball players. The statistical analysis demonstrated that soccer group had a 48% lower level of adiponectin ($P < 0.01$) in respect to basketball players.

Metabolomic analysis using GC-MS

We characterized the metabolic profile of plasma from young athletes, through GC-MS and the identification of each compound was obtained through Fiehn library. Our method allowed us to identify 35 compounds differentially expressed between the two groups of athletes; among them 9 have a statistically different concentration between the two groups. Most of these are amino acids as reported in **Table 2**. In particular as shown in **Figure 2**, in plasma from basketball players the follow amino acids are statistically increased: L-isoleucine (23.6%; $P = 0.003$), L-glutamic acid (16.8%; $P = 0.011$), L-ornithine (13.8%; $P = 0.024$), L-proline (37.0%; $P = 0.0003$), L-valine (17.4%; $P = 0.010$) and L-tyrosine (15.4%; $P < 0.007$). The same upward trend is shown for Urea (19.8%; $P = 0.049$).

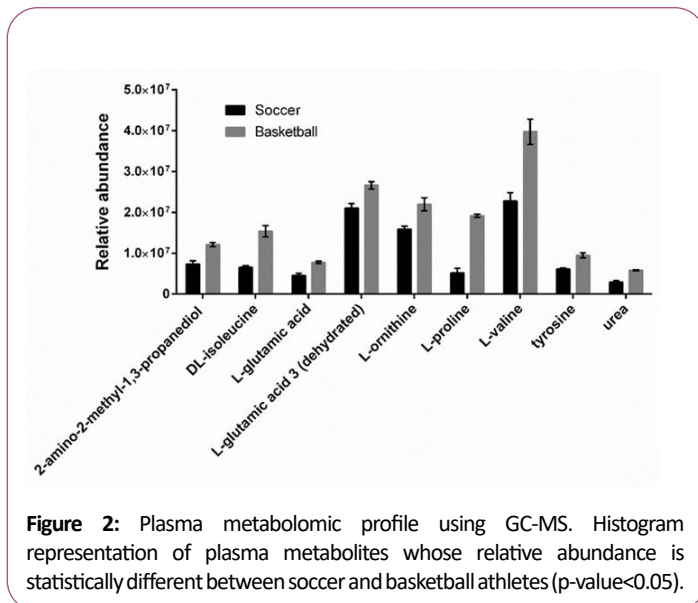


Figure 2: Plasma metabolomic profile using GC-MS. Histogram representation of plasma metabolites whose relative abundance is statistically different between soccer and basketball athletes (p -value < 0.05).

Number	Name	Retention Time	CAS number*	KEGG ID°
Metabolites with significant differences between basketball and soccer players				
1	2-amino-2-methyl-1,3-propanediol	[10.56]	115-69-5	C11260
2	DL-isoleucine	[10.225]	443-79-8	C16434
3	L-glutamic acid 2	[14.398]	56-86-0	C00025
4	L-glutamic acid 3 (dehydrated)	[13.232]	56-86-0	C00025
5	L-ornithine	[16.632]	70-26-8	C00077
6	L-proline	[10.321]	147-85-3	C00148

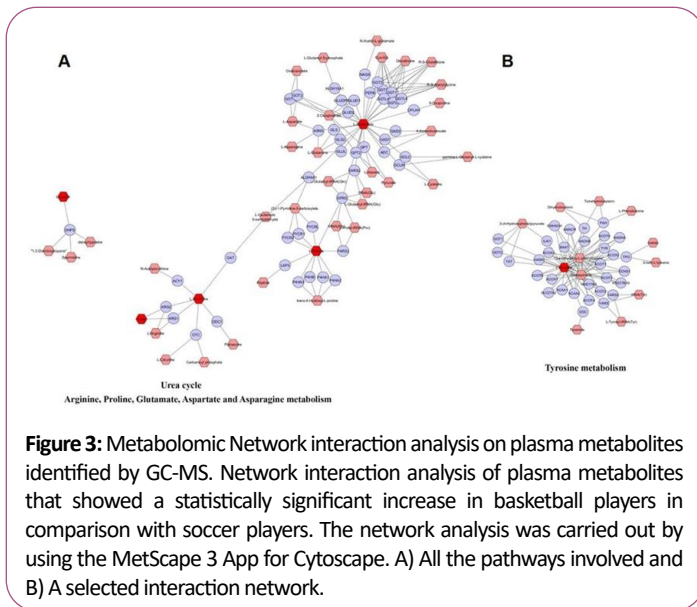
7	L-valine	[9.151]	72-18-4	C00183
8	Tyrosine	[17.871]	60-18-4	C00082
9	Urea	[9.599]	57-13-6	C00086
Metabolites that do not change between basketball and soccer players				
10	1,5-anhydro-D-sorbitol	[16.967]	154-58-5	C07326
11	D-allose 1	[17.278]	579-36-2	C01487
12	D-mannitol	[17.81]	87-78-5	C00392
13	L-sorbose	[17.187]	3615-56-3	C00247
14	L-(+) lactic acid	[6.851]	79-33-4	C00186
15	L-glutamine	[16.092]	56-85-9	C00064
16	L-lysine	[17.643]	56-87-1	C00047
17	L-serine	[11.174]	56-45-1	C00065
18	L-threonine	[11.464]	72-19-5	C00188
19	Acetohydroxamic acid	[7.72]	546-88-3	C06808
20	Citraconic acid	[10.792]	498-23-7	C02226
21	Citric acid	[16.615]	5949-29-1	C00158
22	Eicosapentaenoic acid	[24.013]	10417-94-4	C06428
23	Elaidic acid	[20.508]	112-79-8	C01712
24	Ethanolamine	[9.879]	141-43-5	C00189
25	Glycerol	[9.941]	56-81-5	C00116
26	Glycine	[10.456]	56-40-6	C00037
27	Isopropyl beta-D-1-thiogalactopyranoside	[19.097]	367-93-1	C03619 C02327
28	Methyl-beta-D-galactopyranoside	[16.935]	1824-94-8	C03619
29	Myo-inositol	[19.354]	87-89-8	C00137
30	Oxalic acid	[7.883]	144-62-7	C00209
31	Palmitic acid	[18.846]	64519-82-0	C00249
32	Phosphoric acid	[9.966]	7664-38-2	C00009
33	Threonic acid	[13.652]	7306-96-9	C01620
34	Uric acid	[19.331]	66-22-8	C00366
35	Xanthotoxin	[20.715]	298-81-7	C01864

Note: *Chemical abstract service number KEGG identifier.

Table 2: List of Metabolites identified in plasma of basketball and soccer players by Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

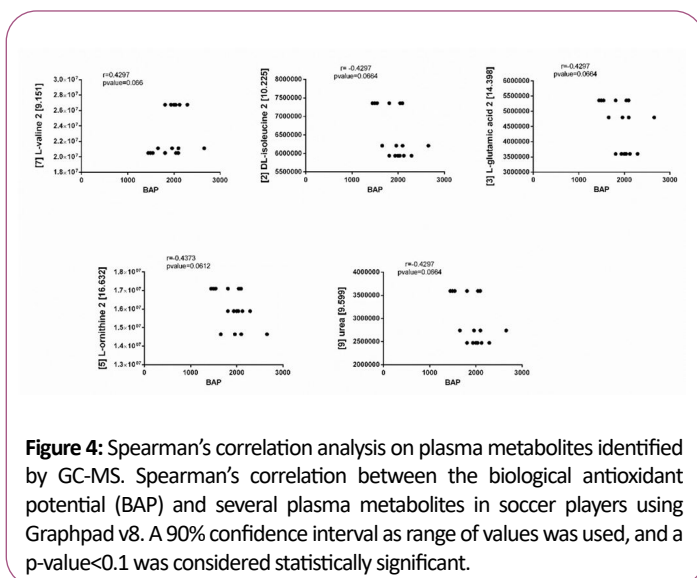
Metabolomic interaction network

We built an interaction networks using the metabolites list obtained from plasma analysis to trace the connections between metabolites and genes and visualize compound networks. We used MetScape which provides a bioinformatics tool for the interpretation of metabolomic data [7]. It analyzes metabolic networks using a database that integrates data from KEGG and EHMN (Edinburgh Human Metabolic Network) [6]. We built the input list using the metabolites that showed a significant increase in plasma from basketball players in comparison with soccer players and reported in **Table 2**. **Figure 3A** reports all the pathways involved and the selected network obtained is showed in **Figure 3B**. It is evident the involvement of urea cycle and metabolism of several amino acids such as arginine, proline, glutamate, aspartate and asparagine (panel A). Moreover, we noted an involvement of tyrosine metabolism (panel B).



Correlation analysis

We determined the Spearman's correlation among adiponectin, the markers of oxidative state (BAP and dROM) with the metabolites that show different concentration in sera between soccer and basketball athletes. For adiponectin, we found no correlation with BAP, dROM and the metabolites analyzed. On the contrary a positive correlation is evident in soccer players between the Biological Antioxidant Potential (BAP) and the aminoacid Valine (p -value=0.066; r =0.43). In contrast, analysing the plasma from soccer players, a negative correlation is evident between BAP with the amino acids Isoleucine (p -value=0.065; r =-0.43), Glutamic acid (p -value=0.065; r =-0.4297), Ornithine (p -value=0.061; r =-0.4373) respectively. Moreover it is interesting to note in soccer players the negative correlation between BAP and urea (p -value=0.066; r =-0.4297). The results are reported in **Figure 4**. No correlation is observed in all athletes among dROM values and plasma metabolites.



Discussion

Physical exercise and even more an intense training is one physical stressor that could cause an imbalance between ROS production and their elimination. The aim of the study is not to evaluate how a specific training programs effects athletes' performance and for this reason the measurement to determine metabolic profile and oxidative stress of athletes were made during "regular season", both for soccer and basketball.

In our study we evaluated oxidative stress status in elite athletes engaged in two sports disciplines: soccer and basketball. In both these sports, the intermittent high intensity activity during matches requires a high function of both aerobic and anaerobic energy systems although in basketball, the aerobic demand is less than soccer [8]. In soccer, power and strength have great impacts over the game that is required during sprinting and in execution of various skills with the ball.

Moreover, these athletes show an increase in marker of muscle damage indicating muscle micro trauma and an increase in markers of inflammation [9]. A basketball match is 40-48 min long and the athletes carry out a combination of high-intensity actions such as sprinting, running, jumping, shuffling with low-intensity activities like jogging and/or walking [10]. The results obtained under our experimental conditions pointed out that in elite athletes with similar BMI, the antioxidant capacity shows a significant reduction in soccer in comparison to basketball players. Moreover, the first athletes show an increase in oxidative species in comparison with the second players. Our data confirm the results of studies previously carried out by other authors performed in soccer players showing an increase in markers of muscle damage as well as an increase in markers of inflammation leads to an increase in ROS that promote post-exercise inflammation [11]. In line with this study, our results show in soccer players a lower plasma level of adiponectin and an increase in reactive oxygen metabolites with a significant reduction in the biological antioxidant potential in comparison to basketball players. Adiponectin secreted and expressed by skeletal muscle [12] is considered a "good adipokine" because its anti-inflammatory effect [13]. Recently the study of adiponectin in myopathies highlights a possible role of the hormone in these diseases [14]. However, there are little data on the relationship between muscular fitness and adiponectin levels independently of several others factors: Adiposity, insulin resistance, dietary patterns and pubertal status. Several authors suggested that adiponectin plasma levels are inversely associated with muscle strength [15]. We are not aware of any study on associations between adiponectin and training that is known to cause inflammation.

Therefore, one of the aims of this study was to evaluate the level of adiponectin in the plasma of athletes who practice two types of sports. In our conditions, we observed a minor adiponectin signal

intensity in soccer players in comparison with basketball players. Plinta et al. [16] observed an increase in adiponectin plasma level after short-term intensive fitness and speed exercise suggesting that the duration and intensity of exercise are important factors that can influence the changes in circulating adipokine levels. Schön et al. [17] found that intense aerobic exercise modulates levels of adiponectin in cerebrospinal fluid suggesting the possibility that adiponectin is exercise-regulated and proposing its implication in a crosstalk between peripheral tissues and brain. In basketball players, we observed an increase in plasma Branched-Chain Amino Acid (BCAA). Increases in serum BCAAs during or after exercise may indicate their mobilization from either liver or muscle [18]. In skeletal muscle they promote glucose uptake and protein synthesis thus playing an important role during exercise and especially in post-exercise recovery. Moreover, exercise increase the ability of mitochondria from skeletal muscle to oxidize BCAAs as an alternative source of energy without inducing insulin resistance [19]. Ammonemia increases during exercise in an intensity-dependent manner and a hyperammonemic state cause central fatigue during exercise through the engagement of the metabotropic glutamate receptors [20]. Urea is not specific to monitor renal function but in professional athletes it might be a signal of a strenuous training session and it could be considered as a recovery parameter and its measurement could be used as an index of adaptation to training.

Serum levels of urea are sometimes used for assessment of training-related stress imposed on athletes because it is a measure of protein catabolism and degradation for gluconeogenesis when the players are prone to overreaching [21]. Urea concentration is a useful biomarker to evaluate athletes' level of fatigue and adaptation to training although a diagnosis of OTS is not possible using this parameter [22]. High levels of urea were found when the athlete shows a high level of performance and the reduction of this plasma urea level is an indication that it is necessary to further increase the intensive training sessions [23]. Moreover, a low concentration of urea indicates the need of an increase in the levels of the exercise load and this value can be therefore used to assess the training load to improve sports performance [24]. In our experimental conditions, we found that basketball players have greater urea and BCAA (including ornithine) concentration suggesting an increase in urea cycle rate to detoxify ammonia. Interestingly, these athletes may have a better system to reduce fatigue and improve exercise endurance capacity associating an increase in urea levels with higher adiponectin concentration and antioxidant capacity [25]. We suggest that it is due to the right training method carried out in this type of sport since adiponectin is produced in the muscles and can therefore have a direct anti-inflammatory effect on the muscle tissue.

Conclusion

Our results highlight that soccer athletes in comparison with basketball, are subject to higher oxidative stress associated with inflammation and metabolic profile alterations. We propose that the high level of adiponectin in basketball players, able to counteract the inflammatory effects of the exercise loads, is due

to the training programme carried out in this type of sport since adiponectin is produced in the muscles and can therefore have a direct anti-inflammatory effect on the muscle tissue.

The significant increase of BCAAs plasma level in basketball players indicates that the intensity and duration of the training induces the ability of skeletal muscle to oxidize BCAAs for energy generation without inducing insulin resistance due to an increase in mitochondria metabolism. Moreover, our data suggest that in basketball players an increase in plasma BCAAs and urea concentrations along with the increase in adiponectin have an effect on athletic performance enhancement because these athletes may have a better system to reduce fatigue and improve exercise endurance. The main limitation of our study is the low number of participants analyzed and therefore we consider this study as a pilot study.

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