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ORIGINAL ARTICLE

Non-invasive approach for the assessment of oxidative stress after intense judo activities

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ABSTRACT

Oxidative stress induced by physical activity has received much attention in recent years due to numerous research papers. Physical activities (aerobic, anaerobic and resistance training) lead to an increased production of highly oxidative substances (RONS, both oxygen and nitrogen containing radicals) and this effect depends mainly on the intensity of muscular work. The combination of anaerobic and aerobic energy systems, as seen in judoka, provides more pathways for free radical production than single exercise training. The purpose of this study was to investigate the use of a non-invasive, simple battery to assess and monitor oxidative stress in judo athletes to better characterize the oxidative stress response to anaerobic and aerobic incremental exercise typical of the judo discipline.

Keywords: Performance analysis of sport, Physical conditioning, Young judoka, Non-invasive sampling, ROS, MDA, Cortisol, Bilirubin.

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INTRODUCTION

Martial arts are physical, mental and spiritual practices, and judo is one of the specialties that require high technical skills and the fastest possible execution of the most important technical actions (Franchini *et al.*, 2011; Thomas *et al.*, 1989). Judo is also described as a multi-joint, high-intensity movement discipline where specific throwing techniques require good physical fitness (Franchini *et al.*, 2003). This dynamic, physically demanding sport requires a high level of physical conditioning and strength to be successful and to compensate for fatigue. Many authors refer to judo as an explosive strength sport that requires tremendous reserves of anaerobic power and capacity, but also operates with a well-developed aerobic system (Callister *et al.*, 1990; Thomas *et al.*, 1989; Ray, 1992).

Considering that a match can last a few seconds or up to eight minutes (5 minutes of play + 3 minutes of golden score), the typical time structure in the match is 20-30 seconds of activity with a 5-10 second pause, during which athletes spend approximately 51% of the time trying to execute a good grip, resulting in a high physiological load on the upper body. Movement time analysis (Sterkowicz, Blecharz and Lech, 2000) has shown that judo competitions are characterized by maximal efforts (100% VO_2) of 10-15 seconds interspersed with recovery periods of submaximal force that include pushing, pulling and lifting. Therefore, both the aerobic and anaerobic metabolic systems are alternately stimulated.

The short burst of energy is provided mainly by anaerobic metabolism, but intermittent muscle work and recovery periods are maintained by aerobic cellular processes (Radovanovic *et al.*, 2009).

It is well described that optimal performance and strength are correlated with the oxidative status of the body. Many studies (Brancaccio et al., 2020; Finaud, Lac and Filaire, 2006; Castrogiovanni and Imbesi, 2012; Nikolaidis *et al.*, 2012; Fisher-Wellman and Bloomer, 2009; Teixeira *et al.*, 2009) have now confirmed that contracting muscles produce radicals and other reactive oxygen/nitrogen species (ROS /RNS) and the relation between oxidative stress and sport is proportional to the intensity of physical exercise. Furthermore, it is now well established that the level of reactive species in skeletal muscle plays a critical role in regulating force production. In fact, there is an optimal redox balance in muscle where the contractile apparatus has the highest force production. In combination with other factors such as growth factors and chemokines, ROS is involved in a cascade of events leading to muscle regeneration and repair (Barbieri and Sestili, 2012), but the local persistence of ROS can lead to muscle damage from oxidative injury. Similarly, ROS, produced during exercise, promotes the formation of new mitochondria, but at higher and persistent concentrations they could attack mitochondria and mitochondrial DNA (mtDNA) and block myogenic differentiation.

The different capacities of ROS are part of the intriguing theory of hormesis (Radak, Chung and Goto, 2008). When present at low concentrations, these reactive species are necessary for basic processes in the cellular environment, but when their concentration exceeds a threshold, deleterious effects on DNA, proteins, and lipids can occur. The body's adaptation to a different workout does not occur without fatigue, but the degree of fatigue is important because extreme fatigue can lead to significant cellular changes. The effect of ROS on muscle force production changes when a high ROS concentration occurs, and the result is a decrease in strength.

The ability of the human organism to prevent or counteract oxidative damage can be analyzed with a series of integrated biomarkers, indicators of normal and pathological biochemical processes. Several in vitro markers of oxidative stress exist, but most are of limited value in vivo because they lack sensitivity and/or

specificity or require invasive methods and expensive equipment. However, it is desirable that these assays are non-invasive sensitivity tests for the effects of exercise on the human body.

The aim of this work is to apply a simple test battery in judo athletes to investigate the oxidative status response to a sports competition and to compare it with that shown in non-competitive effort and in non-effort situations. Each athlete is studied during a microcycle of training/competition, and the reference status is the same athlete at rest. The main objective of the work is to study how judo athletes react to stress experienced in training and competition situations analyzing the optimal redox state of athletes to reduce skeletal muscle damage and be successful in competition.

MATERIAL AND METHODS

Reagents

Bilirubin (BR) (Lot. 031M1429V #B4126; Sigma-Aldrich), dibasic sodium phosphate (Na_2HPO_4), monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$), sodium chloride (NaCl), hydrochloric acid (HCl), pycric acid ($C_6H_2(NO_2)_3OH$), sodium hydroxide (NaOH), sodium tetraborate decahydrat ($Na_2B_4O_7 \cdot 10H_2O$), sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$), glacial acetic acid (CH_3CO_2H), 2,4,6-tripyridyl-S-triazine (TPTZ), Iron(III) chloride anhydrous ($FeCl_3$), thiobarbituric acid (TBA), cortisol standard, dichloromethane (CH_2Cl_2), acetonitrile (CH_3CN), bovine serum albumin fraction V (BSA, A-7906), β-glucuronidase (G8420-25KU) were all analytical grade and was purchased from Sigma-Aldrich. HUG was synthesized and purified as described (Bandiera et al., 2020). Ultrapure water milliQ was used to prepare each solution.

Subjects

Two male judoka who train at the same judo club and compete at the national level volunteered for this study. The physical characteristics of the participants are shown in Table 1. The two athletes were recruited based on their age and judo experience with a frequency of four training sessions (1.5 hours) per week. One of these two athletes was observed one year after the initial observation, during an intense period of competition.

The parents of the participants gave written informed consent to participate in the study, which was conducted according to the protocol approved by the Ethics Committee of the University of Trieste.

Table 1. Subjects physical characteristics.

	Age (years)	Judo practice (year)	Hight (m)	Weight (kg)	Body Mass Index ($kg \cdot m^{-2}$)
Athlete 1	16	9	1.90	116	32.1
Athlete 2	16	9	1.87	89	25.4

Participants guarantee:

1. Good health;
2. No metabolic disorders (diabetes, cardiovascular, hepatic, gastrointestinal or renal disease);
3. No pharmacological treatment or antibiotic;
4. No supplemental vitamin and antioxidants;
5. No smoking;
6. Equilibrate diet;
7. No recent surgery or blood transfusion.

Sampling protocol

Saliva and urine samples were collected in the morning and before/after judo events, as indicated in Table 2. Subjects were instructed to drink almost 1.5 l of water every day. After 1 year, athlete 1 was observed again, during an intense period of competition (Table 2).

Table 2. Saliva and urine monitoring program: the first period panel (A) is related to two athletes (1 and 2), whereas the second period panel (B) is referred to Athlete 1 but two year later.

First monitoring (Judo athlete 1 and 2)	**Second monitoring (Judo athlete 1)***
1. Before normal training*	1. After summer rest time
2. Immediately after normal training	2. After intense training
3. 12 hours after normal training*	3. Before international stage
----------	4. After international stage
4. Before competition*	5. Before/ after an European Cadet Cup
5. Immediately after national competition	6. Before/ after an European Cadet Cup
6. 16 hours after national competition*	7. Before/ after an European Cadet Cup
----------	
7. Before national stage*	
8. Immediately after national stage	
9. 22 hours after stage*	

*Note. * All these specimens were sampled at 8:00 a.m.*

Stimulated saliva samples were collected in a sterile Salivette® (Sarstedt) without any additives. Subjects were instructed not to consume foods or beverages containing sugar, caffeine, or acid for at least 2 hours prior to sample collection. They were also asked not to undergo any dental treatment for at least 48 hours before collection and not to brush their teeth for at least 45 minutes before collection. Before sampling, the mouth is rinsed with water for at least 5 minutes, and then the first saliva produced is swallowed. The sample is collected by placing the Salivette® swab in the mouth and holding it for 2 minutes without chewing. The swab is passed from one side of the mouth to the other and then spat directly into the inner vial of the double chamber tube, sealed and stored at +4 °C until transport to the laboratory. Salivette® were centrifuged at 2000 ×g for 10 minutes to remove particles. Aliquots were immediately stored in vials and placed in the freezer (-20 °C) until analysis. Urine was collected in a sterile 50-mL falcon and transported in a cooler. Saliva and urine samples were rapidly transported to the laboratory and immediately frozen at -80 °C until analysis.

Creatinine concentration in urine

Creatinine concentration was estimated from each urine sample by the Jaffé (Jaffé, 1886) alkaline picrate method. Two reagent solutions were prepared:

Reagent 1: 0.6 g of pycric acid dissolved in 50 Ml of borate buffer (13.6 g of NaOH and 19.83 g of $Na_2B_4O_7 \cdot 10\ H_2O$ at final volume of 1L).

Reagent 2: 1M NaOH solution.

The working reagent was prepared by mixing reagent 1 and reagent 2 (1:1).

Briefly, 40 µL of urine was mixed directly with 360 µL of MilliQ water in a reduced volume spectrophotometer cuvette. Then 2.8 mL of working reagent was added and the mixture was allowed to stand in the dark at room temperature for 10 minutes after shaking. Then the absorbance at 500 nm was read and the creatinine

concentration was calculated using a standard calibration curve.

FRAP assay

The FRAP (Ferric Reducing Antioxidant Power) assay is carried out under acidic conditions (pH = 3.6). The oxidant in the assay is prepared by mixing TPTZ (tripyridyltriazine as the iron-binding ligand), acetate buffer, and $FeCl_3$ and the mixed solution is referred to as "*FRAP reagent*" (Benzie and Strain, 1996). FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue coloured Fe(II)-TPTZ compound from colourless oxidized Fe(III) form by the action of electron donating antioxidants.

Reagents:

1. Solution (A) Acetate buffer, 300 mM, pH = 3.6: add 3.1 g of sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$) in 16 mL of glacial acetic acid (CH_3CO_2H) to final volume 1 L.
2. Solution (B) 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ): add 0.312 g of TPTZ in 100 mL 40 mM HCl. The TPTZ is dissolved at 50°C.
3. Solution (C) 20 mM $FeCl_3$: add 0.360 g of iron(III) chloride anhydrous ($FeCl_3$) in 100 mL of MilliQ water.
4. Working FRAP reagent: mix the solutions A: B: C in the rate (10: 1: 1). The working solution must be freshly prepared.

Aqueous solutions of ascorbic acid in the range of 0.1-0.5 mM are used to calibrate the FRAP assay. The assay procedure requires a freshly prepared working reagent FRAP (3.0 ml) mixed with 100 µl of test sample or standard in a test tube. After stirring, the absorbance at 593 nm is read exactly after 6 minutes against a blank reagent sample.

Malondialdehyde assay

Malondialdehyde (MDA) was determined by the fluorometric method (Agarwal and Chase, 2002), which is based on the reaction of lipid peroxidation (LPO) by-products, such as malondialdehyde, with thiobarbituric acid (TBA) to yield a fluorescent compound.

Derivatization of the urine sample was performed according to the method described by Agarwal and co-workers (2002), and the MDA product was determined using a Dionex HPLC instrument equipped with an AD25 UV-Visual detector and a GF2000 fluorescence detector. The injection volume was 25 µL and the column was a LiChrospher RP-C18 (5 µm, 250 mm, Agilent). The fluorescence detector was set to an excitation wavelength of 515 nm and an emission wavelength of 553 nm.

Cortisol assay

Cortisol is determined in saliva samples according to the proposal of Pihut (Pihut et al., 2015) with some modifications. A certified reference cortisol from Sigma-Aldrich was used as a standard (1.0 mg-mL^{-1} in methanol, vial of 1 mL). Briefly, saliva samples were treated by adding 4 mL of dichloromethane to 1 mL of saliva. Stirred for 10 minutes and centrifuged at 10,000g for 7 minutes. The dichloromethane layer is separated and evaporated. The residue is collected in 100 µL of mobile phase (acetonitrile/water, 36:65 v/v), transferred to an Eppendorf tube, and centrifuged at 4,500 g for 4 minutes. The sample was then injected into the HPLC column (RP-C18) and analysed using an isocratic program at T = 25°C, λ = 240 nm, and a flow rate of 1 mL-min^{-1}.

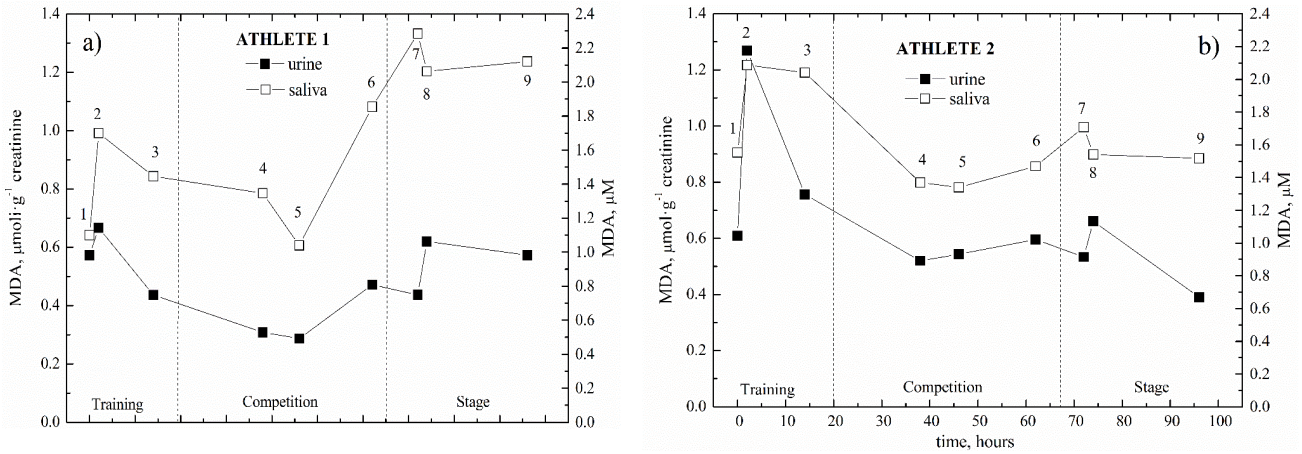
The area under the curve (AUC) for cortisol was calculated using the trapezoidal equations described in Fekedulegn et al. (2007).

Bilirubin fluorometric assay

200 µL of each diluted sample or BR standard solution was added to 10 µL of a HUG 1 g·L^{-1} (PBS, pH = 7.4) in a 96-well microplate (Nunc®) for fluorescence-based assays (Sist et al., 2022). The microplate was then incubated at room temperature for 2 hours, and fluorescence emitted from the BR·HUG complex was detected at λ = 528 nm after excitation at λ = 485 nm using a benchtop microplate reader (Synergy H1; BioTek, Winooski, VT). At this time, 4 units of β-glucuronidase were added, and total bilirubin was detected after overnight incubation at 25°C. The concentration of BR was determined from a calibration curve ranging from 0 to 15 nM. Conjugated bilirubin was determined as the difference between total bilirubin and unconjugated bilirubin.

RESULTS

In a biochemical study, it is recommended to use a series of assays, including different assays for total antioxidant capacity and different assays for oxidative stress markers. In the first part of our study, we examined the correlation between urinary and salivary MDA concentration in saliva and urine samples collected in the morning before and after judo competitions (see Table 2, panel A). Specifically, we wanted to test the hypothesis that salivary MDA concentration correlates with urine levels to provide a simple, reliable, and non-invasive method for determining MDA concentration in athletes during intense training activity and recovery after training. The formation of lipid hydroperoxides by oxidative lipid damage leads to the by-product of lipid peroxidation, such as malondialdehyde (MDA), which is formed by the cleavage of peroxidized fatty acids. MDA is usually measured by derivatization with thiobarbituric acid (TBA), yielding a red compound. The advantages of this method are the relatively short run times and the higher sensitivity and selectivity, especially for urine and saliva samples containing multiple co-elution interferences.



Note. Training: (1) before (8:00 a.m.); (2) immediately after; (3) 12 hours after (8:00 a.m.). Competition: (4) before (8:00 a.m.); (5) immediately after; (6) 16 hours after (8:00 a.m.). Stage: (7) before (8:00 a.m.); (8) immediately after; (9) 22 hours after (8:00 a.m.).

Figure 1. Salivary and urinary MDA concentrations as a function of time, during training/competition event for a) athlete 1 and b) athlete 2. Analyte concentration is divided by creatinine concentration to evaluate the difference in urine dilution.

Comparing the data in Figure 1a with those in Figure 1b, the overall mean shape of the MDA curves in the urine and saliva of the two athletes during the three events was very similar. It is noteworthy that the trends of the two MDA curves (urine and saliva) for each athlete are very similar and have the same range of variation, i.e., 0.6-1.3 µmol·g^{-1} of creatinine for MDA in urine and 1.0-2.3 µM for MDA in saliva. Of particular

note, both saliva and urine values in Figure 1 show high peaks after intense aerobic activities such as a normal club training session (values 1-2-3) or a stage event (values 7-8-9) and, in contrast, lower values during a competition event, as shown in Figure 1. A significant increase in MDA levels (in urine and saliva) measured immediately after the training session (1-2-3) and stage (7-8-9) compared to the corresponding levels before the events is also shown in Figure 1. After a 12-hour recovery, MDA decreased significantly in both the samples.

A correlation plot of MDA in saliva and MDA in urine is shown in Figure 2. The regression shows a linear dependence of MDA in saliva on MDA in urine with a slope of 1.39 and an intercept of 0.81. A good correlation was found with Pearson correlation coefficient, i.e. r = 0.82.

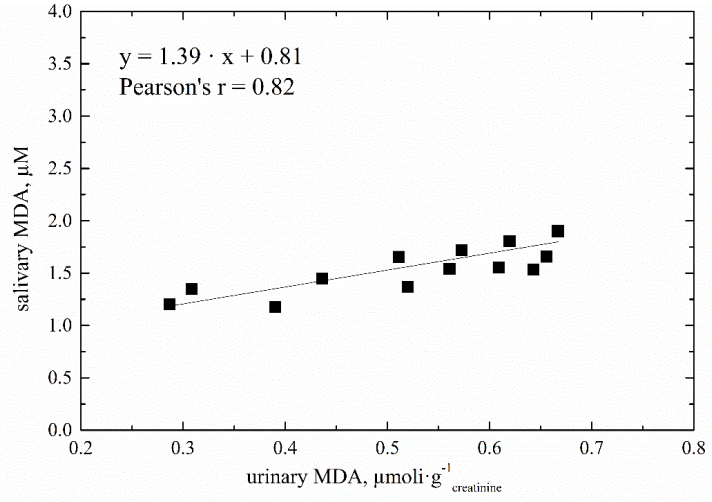


Figure 2. Correlation plot of salivary MDA vs. urinary MDA.

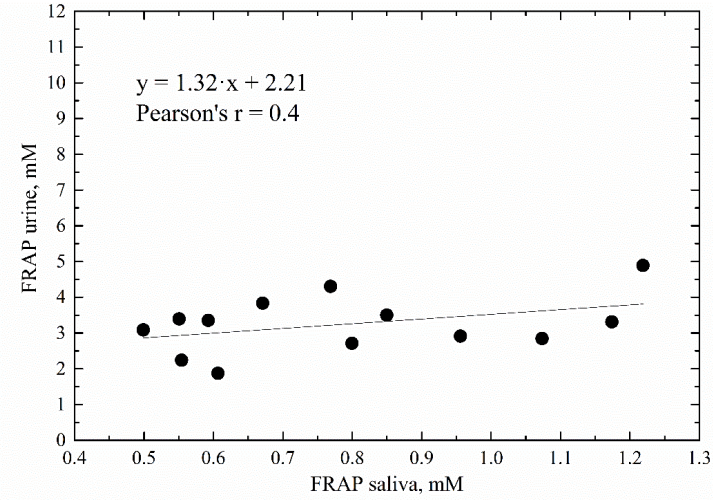


Figure 3. Correlation plot of salivary FRAP vs. urinary FRAP.

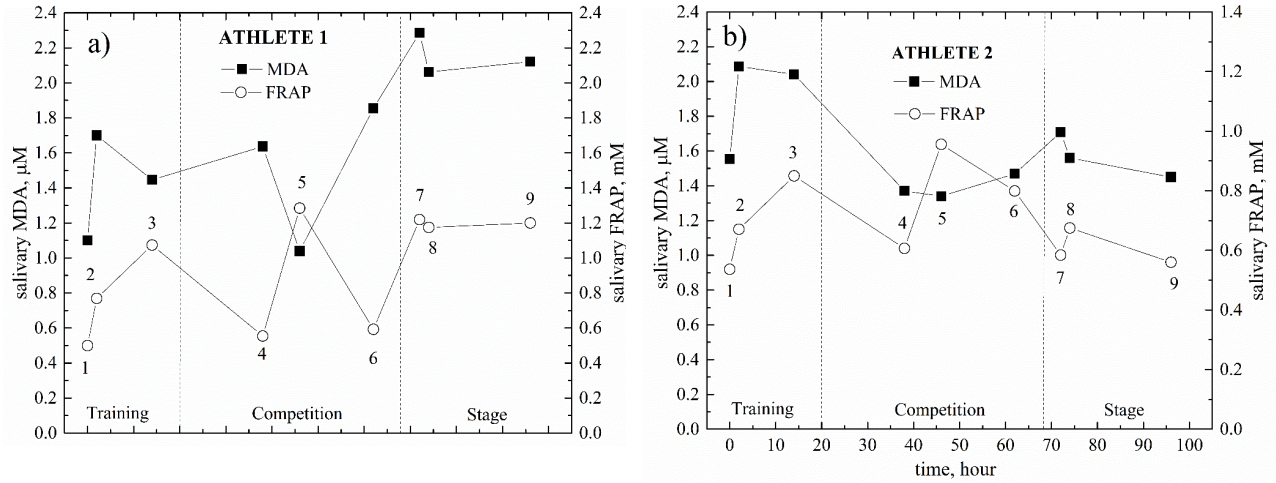
The results of the correlation allow us to monitor the athletes during the different moments of training or competition with greater punctuality and speed, since the implementation is very simple and the samples can

be easily collected, stored and shipped, and they can be obtained at low cost in sufficient quantities for analysis.

The overall oxidative status of athletes was monitored in order to avoid imbalances caused by oxygen free radicals, which are responsible for fatigue status and diseases such as heart failure and muscle damage. In biological samples such as saliva or plasma, there are a large number of heterogeneous compounds with different antioxidant activities that are very difficult to measure separately, so antioxidant status is better represented by total antioxidant capacity as FRAP value.

Figure 3 shows the correlation between saliva and urine FRAP values. As with the MDA parameter, the results of the correlation allow us to monitor the athletes with greater speed and a very simple procedure by taking saliva samples.

In Figure 4 are reported salivary MDA concentrations and salivary FRAP values for athletes in the previous exercise conditions (Figure 1). There was a statistically significant effect of intense exercise on salivary antioxidant capacity FRAP, which increased after training/competition events compared with values measured before the events and decreased after 12 - 22 hours of rest.

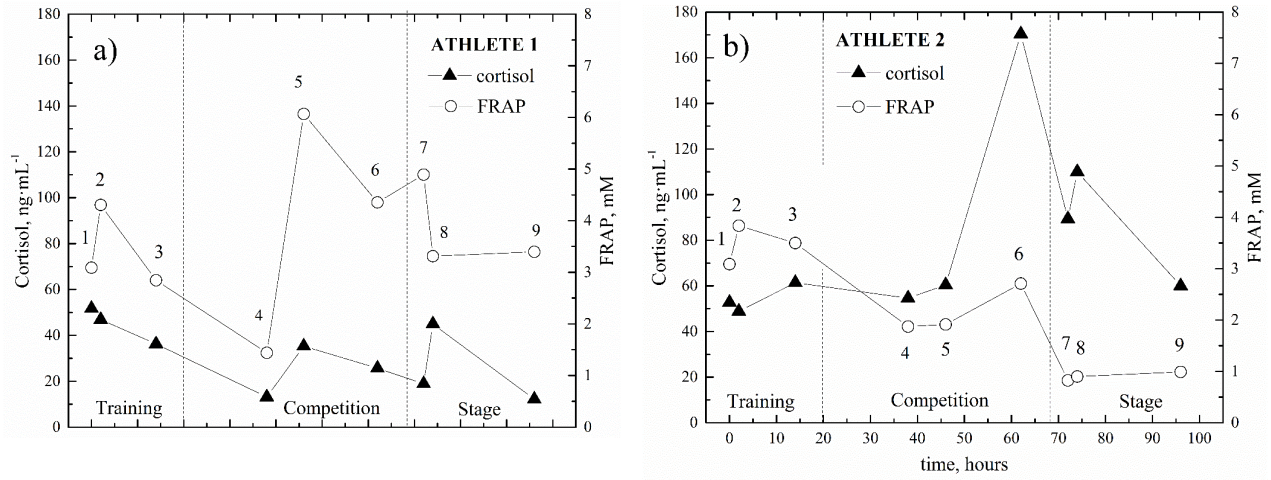


Note. Training: (1) before (8:00 a.m.); (2) immediately after; (3) 12 hours after (8:00 a.m.). Competition: (4) before (8:00 a.m.); (5) immediately after; (6) 16 hours after (8:00 a.m.). Stage: (7) before (8:00 a.m.); (8) immediately after; (9) 22 hours after (8:00 a.m.).

Figure 4. Salivary MDA concentrations and FRAP as a function of time, during training/competition events for a) athlete 1 and b) athlete 2.

Exercise is a physical stressor and activates hormonal systems, especially hypothalamic corticotropin-releasing hormone. In addition to physiological factors, the adrenocortical response to acute or chronic stress exercise is also influenced by psychological factors. Hormones associated with the hypothalamus and pituitary gland, such as salivary cortisol, have been proposed as useful biomarkers of oxidative stress and the decline in physical performance known as overreaching or overtraining. Moreover, salivary cortisol correlates strongly with free blood cortisol ($r \geq 0.90$) and is independent of transport mechanisms and salivary flow (Kaufman et al., 2002). This correlation between salivary and serum oxidative biomarkers reinforces the utility of saliva as a valid diagnostic fluid.

Figure 5 shows the individual responses of salivary cortisol concentration in samples measured during the three events mentioned above.



Note. Training: (1) before (8:00 a.m.); (2) immediately after; (3) 12 hours after (8:00 a.m.). Competition: (4) before (8:00 a.m.); (5) immediately after; (6) 16 hours after (8:00 a.m.). Stage: (7) before (8:00 a.m.); (8) immediately after; (9) 22 hours after (8:00 a.m.).

Figure 5. Salivary cortisol and FRAP concentration as a function of time, during training/competition event for a) athlete 1 and b) athlete 2.

It is noticeable that total cortisol levels in Figure 5 show the same overall trend as antioxidant capacity, expressed by measurements from FRAP, which increase immediately or within 12 hours after competition and national stage events. It is well known that cortisol levels increases linearly in response to physical exercise until a specific threshold of exercise intensity is reached, with peak concentrations generally occurring at the end of physical activity (Ahtiainen et al., 2003; Filaire et al., 2001).

The values measured on the day of the competition (value 5 and 6 in Figure 5) were significantly higher than the values measured after normal training events. Significant cortisol differences were noted near the end of the competition, which has already been reported to increase stress, as measured by higher salivary cortisol levels (Moreira et al. 2013; Filaire et al. 2001) during judo competition, confirming previous reports of the existence of an anticipatory cortisol response to this type of situation. The peak cortisol increase is reported to occur at 20 min post-exercise in blood and at around 30 min post-exercise in saliva (Crewther et al., 2008; O'Connor et al., 1987). This response is similar to that found for other sports such as volleyball and basketball observed during an important match (Mazdarani et al. 2016; Moreira et al., 2013).

It is noticeable that the response of the two athletes is very different in terms of cortisol levels, with athlete 1 always having a lower cortisol salivary concentration (Figure 5).

The interindividual variability of cortisol levels measured in the pre-post events of Figure 5 is also shown as the change in the area under the curve (AUC) values in Table 3. The AUC is routinely used to account for multiple time values in monitoring over a period of time. Following Pruessner et al. (2003), the area under the curve was calculated using the trapezoidal formula to obtain the area under the curve with respect to rise (AUC_I) and the area under the curve with respect to ground (AUC_g). AUC_I is calculated with respect to the baseline (starting value) and highlights the changes as a function of time, while AUC_g expresses the total hormone release. In the repeated measurements. AUC_I takes into account both the sensitivity and intensity

of changes in the pattern and takes both negative and positive values in terms of the shape and size of the curve, as in Table 3. Therefore, for patterns that have a sequence of increases and decreases over time, the AUC_I can be positive or negative depending on how much the increase is relative to the decrease.

Significant differences in the values of AUC_g were found between training event and competition as well as between competition and stage training as reported in Table. 3. These data shows that the value of AUC_g in the competition situation was higher than in the training situation in both athletes, namely from 597 to 681 (+14%) and from 762 to 2306 (+203%) in athlete 1 and 2, respectively. The competition situation may represent more psychological demand of athletes and influenced their perception of the difficulty and importance of the match. Moreover, athlete 1 showed an almost constant AUC_g value throughout the entire observation period, whereas athlete 2 showed an increase in AUC_g value of up to 200% during the competition. In addition, athlete 2 showed significantly higher AUC_g values than athlete 1. As reported in the literature, AUC_g and performance are related, with the greatest performance occurring in athletes who have low AUC_g values (Meggs et al., 2016). Therefore, AUC_g can be adopted as a measure to predict athletic performance in competition and may be useful in monitoring athletes' overall exposure. These differences can be better observed comparing the AUC_I values in Table 3, where the AUC_I is calculated with reference to the initial AUC value. Analysis of pre-post changes in cortisol levels showed high interindividual variability, which allowed us to distinguish between athletes with different HPA axis responses and adaptation to the same training load showing an increase, unchanged, or decrease in AUC_I levels.

Table 3. AUC cortisol data from first monitoring for athlete 1 and athlete 2.

Athlete 1		**Athlete 2**		
AUC_g	AUC_I	AUC_g	AUC_I	
597	-126	762	-25	1. Before normal training (8:00 a.m.) 2. Immediately after normal training 3. 12 hours after normal training (8:00 a.m.)
681	366	2306	996	4. Before competition (8:00 a.m.) 5. Immediately after national competition 6. 16 hours after national competition (8:00 a.m.)
691	238	2069	-86	7. Before national stage (8:00 a.m.) 8. Immediately after national stage 9. 22 hours after stage (8:00 a.m.)

Athlete 1 was then observed after one year when he additionally participated in higher level competitions, such as international competitions. Table 4 shows the data on MDA, cortisol, and bilirubin in saliva before and after competitions and training events. Bilirubin was included in our test battery as a preliminary outcome because it has widely been used in sports medicine to assess the degree of muscle damage or strain leading to haemolysis (Banfi et al., 2012.), but its contribution to the total antioxidant capacity of human blood is recently underlined (Witek et al., 2017).

Table 4 shows higher MDA values in the second period (B) compared to period A, which is likely due to the higher training load and volume in this year, especially during competitions.

Very low or no cortisol levels were detected during the period B training and stage events, mainly attributed to the current training and fitness status of athlete 1. In contrast, cortisol levels were significantly higher after the competition 3 than before the competition (Table 4), most likely due to cognitive or somatic anxiety: fear, panic, alarm, anxiety, apprehension, and symptoms caused by autonomic nervous system activation, such

as accelerated heartbeat, sweating, etc., in cognitive anxiety situations (Hardy et al., 2009) confirming the anxiogenic nature of sports contests.

Table 4. Stress biomarkers for athlete 1 at two different moments (A and B) of his judo career.

Event monitored		Training		Stage		Competition 1		Competition 2		Competition 3	
		A	B	A	B	A	B	A	B	A	B
MDA	Before	0.572	1.413	0.437	1.040	0.308	0.996	--	1.442	--	1.286
µmol·g^{-1}	After	0.436	1.007	0.573	0.628	0.471	1.200	--	0.921	--	0.999
Cortisol	Before	51.70	0.00	18.90	0.00	13.11	0.00	--	1.76	--	22.66
ng·mL^{-1}	After	36.27	16.20	12.15	0.00	25.65	0.81	--	0.00	--	28.87
T-BR	Before	--	1.47	--	0.972	--	--	--	2.193	--	0.735
nM	After	--	--	--	2.295	--	0.609	--	0.626	--	2.871
BR	Before	--	0.547	--	0.851	--	--	--	1.142	--	0.478
nM	After	--	--	--	1.365	--	0.413	--	0.152	--	0.429
C-BR	Before	--	0.925	--	0.120	--	--	--	0.478	--	0.256
nM	After	--	--	--	0.930	--	0.196	--	0.429	--	2.442

Note. T-BR: total bilirubin, BR: bilirubin, C-BR: conjugated bilirubin.

Bilirubin has also been measured in salivary samples as an antioxidant and anti-inflammatory biomarker, as it can act as an antioxidant defence against ROS attacks and its level can be stabilized by a good exercise protocol. Our preliminary results on all three bile pigments in athlete 1 (Table 4) shows a correlation between salivary bilirubin and cortisol. The role of free and conjugated bilirubin found in saliva deserves further investigation and correlation with changes in plasma bilirubin.

DISCUSSION

In the present study, a number of oxidative stress biomarkers were examined before and after a training, stage, or competition situation. The main findings of the current study were that aerobic and anaerobic disciplines such as judo can cause an increase in oxidative stress when the antioxidant system is inefficient in response to additional free radical production during exercise. However, when training programs are long and intense enough to establish a consistent adaptive response of the antioxidant system, a decrease in oxidative stress occurs. It can be concluded that regular judo training may serve as a stimulus to enhance endogenous antioxidant protection in judoka through their regular and strenuous training. Routine monitoring of judo athletes requires only non-invasive sampling performed immediately after training or competition. In addition, the ability to store biological samples at low temperature prior to analysis without compromising the determination of chemicals is critical for the study of elite athletes in various geographic locations. We have shown that the response of athletes to intense training can be measured in a reliable and non-invasive manner by serial saliva sampling, in part because the transfer of markers from blood to saliva is relatively rapid, for example, cortisol within 2-3 minutes. In addition, the monitoring procedure is simple and applicable under a wide range of training and competition conditions. Moreover, the procedure avoids the need to draw blood samples from the vein, which is associated with additional stress and can lead to false positive results.

From the preliminary results obtained here, it appears that the two athletes considered respond very differently to the tests, despite having the same training and experience. Thus, in monitoring oxidative and psychophysiological stress, each individual is a reference in itself and the average over a population of athletes may not be representative. FRAP assays have the advantage of being able to account for the

individual antioxidant effects of different substances and their additive, synergistic, or antagonistic interactions. Increased antioxidant capacity need not be a desirable state if it represents a response to increased oxidative stress. Similarly, a decrease in antioxidant capacity may not necessarily be an undesirable state if the measurement reflects decreased production of reactive species.

Salivary cortisol is a representative marker of circulating free cortisol and can be used as an index of exercise stress while avoiding venepuncture-induced stress. Therefore, cortisol is used to measure psychophysiological stress during single or repeated training sessions or competitions by relating the intensity of anxiety and salivary cortisol concentration before and after the competition. Another aspect to consider is that cortisol taken and measured from serum or plasma represents total cortisol and not free, biologically active cortisol.

The differences in cortisol levels found in this work may be due, at least in part, to differences in physical exertion, but the psychological stress of competition contributes significantly to the physiological stress caused by exertion. It is important to remember that stress hormones such as cortisol cause immune suppression and resistance to infection decreases in athletes after intense and prolonged exercise. In the present study, individual data showed significant inter-individual variability in pre- and post-exercise cortisol level changes and area under the curve (AUC) values. This observed variability is the result of the different response of the hypothalamic-pituitary-adrenal (HPA) axis to exercise stress, which leads to a different physiological adaptation of the neuroendocrine system of athletes. These data relate to the individual response to the stressor and suggest that the individual monitoring protocol is preferable to smoothed averages of the athlete population. In addition, monitoring hormonal response to exercise may be a useful indicator of excessive fatigue and the onset of overreaching or overtraining status. A significant increase in cortisol levels at rest indicates the onset of overtraining.

Cognitive anxiety has been shown to take on high values before a competition and to remain relatively high and stable at the beginning of the competition. Somatic anxiety, on the other hand, has relatively low values until about 12-24 hours before the competition. Thereafter, a rapid and significant increase is observed as the start of the competition approaches. Throughout the duration of the competition, cognitive anxiety and, consequently, cortisol levels vary as a function of the probability of success/failure, whereas a rapid decline was observed in somatic anxiety levels. In addition, several studies reported that cortisol levels varied between winners and losers, with losers having higher cortisol levels, especially immediately after competition (Fernandez-Fernandez et al., 2015).

Bilirubin has been shown to be a molecule with multiple functions. In the past, it was a degradation product with toxic effects, especially in infants. In recent years, it has assumed various physiological meanings, and important antioxidant, anti-inflammatory, and regulatory effects have been attributed to it. The evaluation of the baseline level of bilirubin in saliva and its changes after stressful situations is an innovative measure that is not currently used because the concentrations are very low and cannot be detected by conventional analytical methods.

This simple salivary test battery for oxidative and psychological stress needs to be complemented by other stress biomarkers such as amylase, testosterone, lactic acid, IgA/IgM, enzymatic activities (SOD, peroxidase), uric acid, which would be particularly useful out of competition during a period of reduced training, such as during a recovery period or during a taper, where it is important to determine the psychophysiological profile of an athlete in preparation for competition.

The analysis of pre-post changes in cortisol level showed the existence of a high-interindividual variability which allowed us to distinguish between athletes with different HPA axis adaptation to the same training load. This work encourages further research into the effects of the training program in athletes to prevent fatigue and injury and to maintain the health of these athletes.

CONCLUSIONS

Heart failure and muscle damage are two phenomena that affect professional and elite athletes. In particular, heart failure occurs in 1/100,000 athletes, while the incidence of muscle injury in athletes is about 30-40% of total injuries. In addition, fatigue in athletes is a common but difficult to diagnose condition. While some degree of fatigue may be normal in any athlete during periods of high training volume, it is very important for sports physicians to distinguish between this physiological fatigue and more prolonged and severe fatigue, which is often due to certain pathological conditions. It is well known that stress is one of the factors highly associated with the possibility of injury. During stressful periods, muscle strains and minor injuries are more frequent, even during simple activities.

In summary, it seems possible that the ROS- and RNS-induced reduction in maximal force production may be part of a protective mechanism by which skeletal muscle protects itself from further peak force-generated damage. In addition, it has been suggested that repetitive muscular ROS-induced fatigue combined with inadequate recovery may trigger an overtraining syndrome.

Aerobic, anaerobic, or mixed exercise causes increased production of free radicals. Similarly, humans respond adaptively with increased mobilization of a variety of enzymatic and non-enzymatic antioxidants in cells or plasma. However, in most cases, antioxidant capacities are exceeded, leading to oxidative stress, which is even more important when training intensity and duration are high and subjects have low training levels and inadequate nutritional status. This exercise-induced improvement in antioxidant status and reduction in oxidative stress have been extensively documented in the literature. However, some studies report a decrease in the efficiency of the antioxidant system, particularly in high-performance athletes exposed to high training and competition loads with inadequate nutrition. These studies suggest a threshold at which oxidative stress may increase excessively and lead to overtraining. Indeed, the free radicals generated during exercise play an important role in the development of muscle damage, but also in the development and spread of post-exercise inflammation, which can exacerbate cellular damage. The totality of these phenomena can be the cause of muscle fatigue and injury, disrupting muscle function and leading to overtraining syndrome.

AUTHOR CONTRIBUTIONS

Paola Sist: Sample collection, Methodology, Investigation, Writing-original draft, Result and Discussion. Ranieri Urbani: Methodology, Formal Analysis, Software, Writing-original draft, Critical Revision and Discussion, Review and Editing.

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