Seasonal Longitudinal Study of Oxidative Stress and Redox Status in Professional Athletes of Different Sports

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ABSTRACT | It is well established that physical exercise induces reactive oxygen species production, while physical training increases antioxidant defense systems thereby reducing exercise-induced oxidative stress. However, intense physical exercise, competition, and training such as those imposed on high-level athletes may lead to increased oxidative stress implicating an overtraining condition. In the present study, the effects of training and competition load on oxidative stress, redox and antioxidant status, and cell damage markers have been studied on high-level players in different sports (i.e., 15 ultra-endurance and 12 long-distance runners, and 12 junior professional rugby players) during a competitive season. Oxidative damage plasmatic markers, including protein carbonyls (PC) and thiobarbituric acid-reactive substances (TBARS), total antioxidant capacity (TAC), and amino-thiols redox status were assessed at three critical time points of the season: T0, the beginning; T1, the top; T2, the end. Seasonal variations of all the investigated parameters were observed. With respect to the basal level T0 (0.69 ± 0.14), significant increases of PC concentration (nmol/mg protein) at T1 (1.08 ± 0.36, $p < 0.001$) and T2 (1.04 ± 0.27, $p < 0.01$), as well as of TBARS concentration (µM, T0: 6.11 ± 1.11) at T2 (8.09 ± 1.07, $p < 0.0001$) and T1 (6.77 ± 1.22, $p < 0.01$) were measured in ultra-endurance athletes. PC was found significantly increased in rugby players too (T0: 0.76 ± 0.26 vs. T2: 1.19 ± 0.34; $p<0.05$). With regard to the oxy-redox status, TAC activity was not found significantly changed during the monitored time period, while amino-thiols redox status was upregulated, as demonstrated by the increase of oxy and total glutathione (GSH) and cysteine (Cys) concentrations, particularly in ultra-endurance athletes. On the other hand, the significant decrease in reduced GSH, Cys, and cysteinylglycine (CysGly) concentrations measured in rugby players at T2 suggested that non-enzymatic antioxidants were strongly consumed during the competitive season, and this finding was closely linked to an overtraining condition. Altogether, the results achieved in the present study confirmed the hypothesis that oxidative stress and antioxidant measurements play a peculiar role in monitoring the athletes' training response and performance, whose variations are in turn strictly linked to the played activity.

KEYWORDS | Long-distance runners; Oxidative stress; Rugby players; Thiols redox status; Total antioxidant capacity; Ultra-endurance runners

RESEARCH ARTICLE

ABBREVIATIONS | Cys, cysteine; CysGly, cysteinylglycine; GSH, glutathione; Hcy, homocysteine; PC, protein carbonyls; TAC, total antioxidant capacity; TBARS, thiobarbituric acid-reactive substances

CONTENTS

1. INTRODUCTION

It is well established that physical exercise induces reactive oxygen species (ROS) production as a consequence of an imbalance between pro-oxidant factors and antioxidant defenses, while physical training increases the antioxidant defense system accompanied by a decrease in exercise-induced oxidative stress [1]. However, due to repeated exercise bouts and also physically demanding practice sessions, professional sport training is expected to produce opposite responses. An excessively stimulated adaptive effect associated with an imbalance between training loads and recovery may lead to an overtraining state [2], wherein the failure of antioxidant systems to adapt to training leads to antioxidant protein damage and depletion of cellular oxidant-scavenging stores. According to the played sport, the physiological load can differ, going from a prevailing continuous steady-state exercise, such as in running disciplines, up to a highly intensive intermittent exercise in team sports like rugby. The specific characteristics of the played sport are expected to lead to different ROS production levels and cellular damage, and the latter is estimated by measurement of oxidative damage biomarkers. In particular, protein carbonyls (PC), derived from albumin or other plasmatic protein oxidation, can provide oxidative damage estimation, while thiobarbituric acid-reactive substances (TBARS) are markers of cell membrane oxidative degradation due to peroxidation of unsaturated fatty acids. On the other side, in order to pre-vent exercise-induced oxidative stress, the organism is well equipped with an antioxidant defense system including total antioxidant capacity (TAC) and non-enzymatic substances such as thiols [3]. In the plasma, aminothiols interact via redox and disulfide exchange reactions, generating a dynamic system referred to as redox thiol status that, by regulating cellular homeostasis, is a critical determinant of cell function [4]. All these reasons lead us to consider oxidative stress biomarkers and TAC measurements, together with the detection and quantification of plasma aminothiols redox status, which are well indicated for overtraining diagnosis due to their association with exercise-induced tissue trauma and injury severity. In the present study, the effects of training and competition load on oxidative stress damage markers, and redox and antioxidant status were studied on high-level players in different sports (i.e., ultra-endurance or long-distance run and rugby) during a competitive season. The final aim of the study was to identify the criteria for an individual training program optimization mainly by preventing non-functional over-reached states.

2. MATERIALS AND METHODS

2.1. Subjects

Three different groups of elite athletes participated in this study. Subjects were selected to form groups homogenous for both training and performance as

follows: (1) Fifteen ultra-endurance (> 50 km) athletes (8 females: age of 39.87 ± 3.89 years, height of 163.40 ± 6.40 cm, body mass of 52.34 ± 3.93 kg, body mass index [BMI] of 19.61 ± 1.19 kg/m^2; and 7 males: age of 40.93 ± 6.07 years, height of 173.33 ± 3.68 cm, body mass of 70.60 ± 1.50 kg, BMI of 23.51 ± 0.58 kg/m^2). All the participants were endurance national and international level high-trained non-professional athletes belonging to Italian Ultra-Marathon and Trail Association team, fully familiarized with ultra-marathon running. All subjects showed 4.50 ± 2.50 years of ultra-endurance experience and followed their own individual preparation program playing their endurance activity by working out 4–5 times a week with a mean of 75 ± 10.50 km/week; (2) Twelve male long-distance (20–50 km range) runners (age of 23.29 ± 5.27 years, height of 175.90 ± 6.50 cm, body mass of 62.90 ± 5.50 kg, BMI of 20.32 ± 0.62 kg/m^2) belonging to regional, national, and international teams. All runners had participated in at least one important international competition and played their endurance activity by working out 4–5 times a week with a mean of 17.10 ± 7.00 h/week; (3) Twelve male juniors professional rugby players (age of 18.85 ± 0.38 years, height of 185.40 ± 6.60 cm, body mass of 95.30 ± 9.80 kg, BMI of 27.63 ± 1.01 kg/m^2) belonging to the same team playing in the top professional Italian and European rugby championship. The training program was set by the team coaches and was not influenced by the experimental study. The average weekly training volume was 9.50 ± 1.00 h: physical fitness (40%) and technical or tactical training (60%). In the first phase of competitive season, training intensity was declared low, then it became high. The team played matches during all the examined periods. Procedures were in accordance with the Declaration of Helsinki, and institutional review board ethical approval was received. Subjects were informed of the nature of the study, and all subjects signed informed consent forms. All of the participants were non-smokers, had no history of medical disorders, and had not taken antioxidant supplements for at least 6 months prior to the study. Moreover, they were instructed to refrain from making any drastic changes in diet.

2.2. Samples

In the time course of the study (one year), blood samples were collected three times (see **Figure 1**) as follows: (1) T0, at the beginning of the seasonal competitive period; (2) T1, at the top of the competitive period; and (3) T2, at the end of the season. All the measurements were carried out under different conditions of training volume and intensity. All the participants were refrained from training, competitions, or other forms of exercise at least 36 h before each data collection point. Blood samples were collected in the morning (9 a.m.). Approximately 15 ml of blood were drawn from an antecubital vein and collected in heparinized (5 ml) and ethylenediaminetetraacetic acid (EDTA) (10 ml)-treated vacutainer tubes (Becton Dickinson and Company, UK). The plasma was separated by centrifuge (5702R, Eppendorf, Germany) at 3000 *g* for 5 min at 4°C. Samples of plasma were then immediately stored in multiple aliquots at –80°C until assayed.

2.3. Analytical Procedures

2.3.1. Determination of Oxidative Stress Biomarkers

All plasma samples were assessed by enzymatic methods using a microplate reader spectrophotometer (InfiniteM200, Tecan, Austria). All measurements were performed in duplicate. The inter assay coefficient of variation was in the range indicated by the manufacturer of the assay kit.

Plasma total antioxidant capacity (TAC) was estimated by using an enzymatic kit (Cayman Chemical, Ann Abor, MI, USA). The assay is based on the ability of plasma antioxidants to inhibit the oxidation of 2,2′-azinobis (3-ethylbenzothiazoline) sulfonic acid (ABTS) to ABTS$^+$ radical cation by a peroxidase. The ABTS$^+$ amount was estimated by measuring the absorbance peak at 750 nm. The concentration of antioxidants is proportional to the suppression of absorbance signal. The TAC absolute concentration was evaluated by a trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard curve and expressed as trolox-equivalent antioxidant capacity (mM).

Oxidized proteins accumulation was measured by determining the reactive carbonyls content. A protein carbonyl (PC) assay kit (Cayman Chemical) was used to spectrophotometrically evaluate the signals at 370 nm. The obtained values were normalized to the total protein concentration in the final pellet (absorbance reading at 280 nm), in order to take into account

the protein loss during the washing steps, as suggested in the kit's user manual.

Thiobarbituric acid-reactive substances (TBARS) were determined to estimate lipid peroxidation. A TBARS assay kit (Cayman Chemical) was adopted which allows a rapid spectrophotometric detection of the thiobarbituric acid-malondialdehyde (TBAMDA) adduct at 532 nm. A linear calibration curve was built up by measuring the signals from pure malondialdehyde containing samples.

2.3.2. Determination of Thiols

Total (tot), reduced (red), and oxidized (oxy) aminothiols were measured in the plasma according to previously validated methods [5]. Briefly, Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) and 4-fluoro-7-sulfamoylbenzofurazan (ABD-F) were used as reducing and derivatizing agents, respectively; reduced aminothiols were assessed by mixing the erythrocytes and the plasma with 10% trichloroacetic acid (1:1 v/v). NaOH (0.4 M, 10 µl), borate buffers (1 M, pH 11, 70 µl as well as 1 M, pH 9.5, 30 µl), each of them containing 4 mM EDTA, and ABD-F (10 g/L, 10 µl, in borate buffer pH 9.5) were added to 100 µl of each of the obtained supernatants. Samples were incubated at 4°C for 90 min and then 10 µl were injected into a high-performance liquid chromatography (HPLC) system for analysis. Thiols separation was performed at room temperature by isocratic HPLC analysis on a Discovery C-18 column (250 × 4.6 mm I.D, Supelco, Sigma–Aldrich, St. Louis, MOS, USA), eluted with a solution of 0.1 M acetate buffer, pH 4.0: methanol, 81:19 (v/v), at a flow rate of 1 ml/min. Fluorescence intensities were measured with an excitation wavelength at 390 nm and an emission wavelength at 510 nm, using a fluorescence spectrophotometer (Jasco, Japan). A standard calibration curve was used. The concentration of the oxidized forms was obtained by calculating the difference between the total and the reduced forms.

2.4. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism package (GraphPad Prism 7, GraphPad Software Inc., San Diego, CA, USA). Data were expressed as mean ± SD. Experimental data were analyzed using repeated Shapiro–Wilk test and compared by variance analysis, ANOVA, with Bon-ferroni multiple comparison test to further check the significance among groups. A $p < 0.05$ value was considered statistically significant.

3. RESULTS

The concentration values of oxidative damage biomarkers obtained at T0, T1, and T2 from all the examined categories of athletes are displayed in **Figure 2**. No significant differences in the reported parameters among the athletes' categories were observed at T0. In ultra-endurance athletes, a significant increase in PC concentration (nmol/mg protein, **Figure 2A**) with respect to the T0 level (0.69 ± 0.14), was observed at both T1 (1.08 ± 0.36, $p < 0.001$) and T2 (1.04 ± 0.27, $p < 0.01$). At the same time, a significant ($p < 0.05$) increase in the PC concentration (T0 $= 0.76 \pm 0.26$ vs. T2 $= 1.19 \pm 0.34$ nmol/mg protein) was observed in rugby players too, while the PC increase measured in long distance runners was not statistically significant. The TBARS concentration (µM) (**Figure 2B**) significantly increased at T2 (8.09 ± 1.07) vs. T0 (6.11 ± 1.11, $p < 0.0001$) and also at T2 vs. T1 (6.77 ± 1.22, $p < 0.01$) in ultra-endurance athletes, while the increases measured in both long distance runners and rugby players were not found statistically significant. As regard as the antioxidant parameters, TAC concentration (mM Trolox) values (**Figure 2C**) were slightly increased at T1 with respect to T0 level in ultra-endurance, and were slightly decreased in long-distance runners and rugby players, but the changes were not statistically significant.

The redox status in the plasma, determined during the season by measuring the concentrations of aminothiols in the total, oxidized, and reduced forms, from all the athlete groups is displayed in **Figure 3**. At T0, significant differences among the three athlete groups were calculated. Total and oxidized GSH concentrations (µM; **Figure 3A1** and **3A2**, respectively) were significantly ($p < 0.0001$) lower in the athletes of running specializations (ox-GSH: ultra-endurance, 4.76 ± 1.44; long-distance, 14.33 ± 1.60) when compared to the rugby players (22.06 ± 2.21). A significant ($p < 0.0001$) difference between ultra-endurance and long distance runners was also observed. Similarly, in terms of Cys forms, significantly lower values were observed at T0 when comparing long distance runners to ultra-endurance
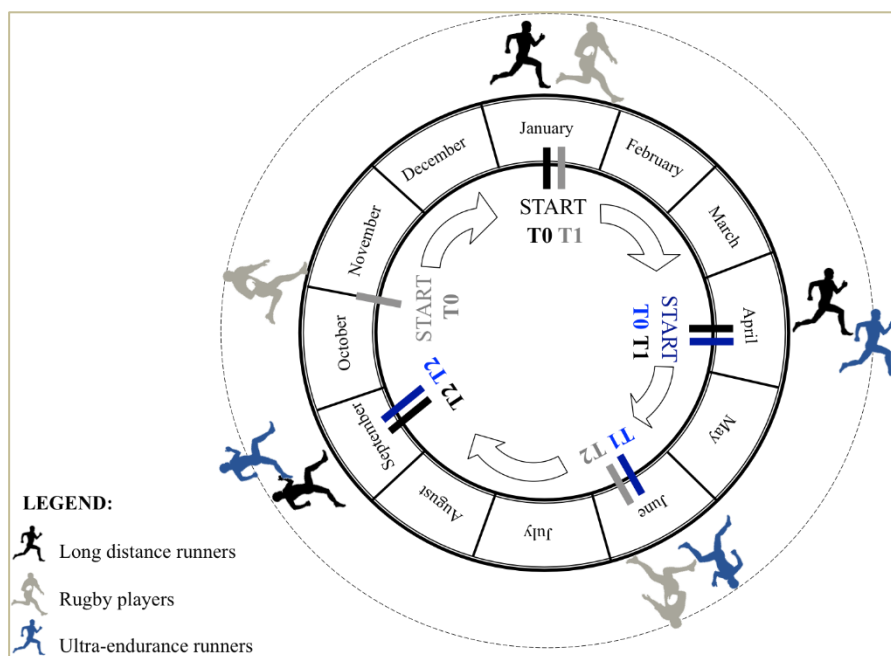
FIGURE 1. Study protocol design and seasonal circle. Ultra-endurance (blue) and long-distance (black) runners and rugby players (grey) were tested three times across a competitive season: at the beginning (T0); at the top (T1); at the end (T2).

athletes (tot: p < 0.05, **Figure 3B1**) and to rugby players (tot: p < 0.0001, **Figure 3B1**; ox: p < 0.0001, **Figure 3B2**; and red: p < 0.0001, **Figure 3B3**). Significantly (p < 0.0001) lower reduced Cys values were also observed at T0 between ultra-endurance athletes and rugby players.
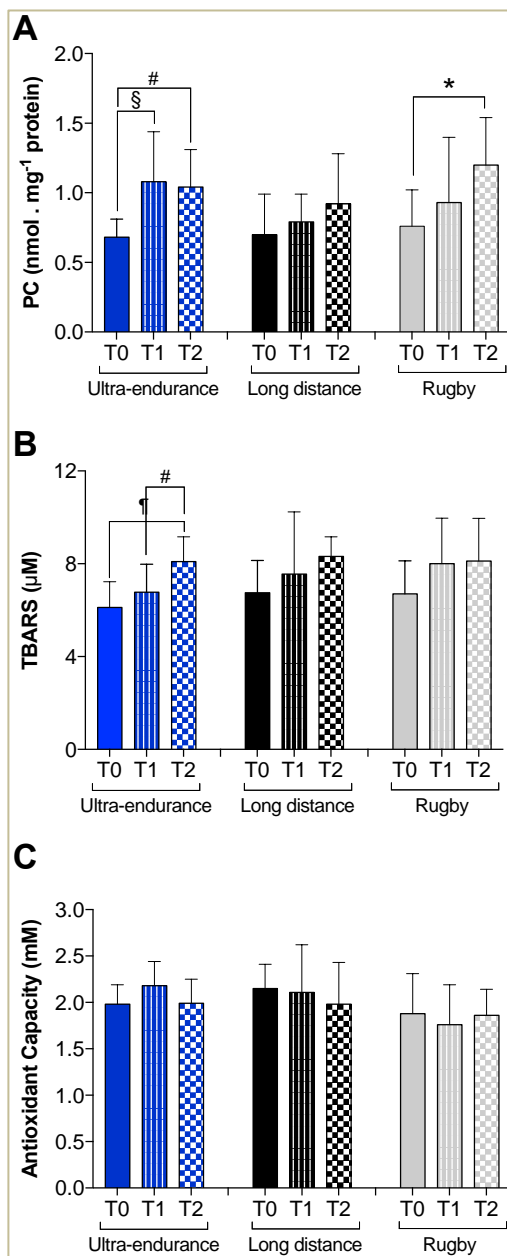
Statistically significant changes were measured in GSH concentrations at the different seasonal times in all athletes' categories (**Figure 3A1**, **3A2**, and **3A3**). Total and ox-GSH (**Figure 3A1** and **3A2**) concentrations were always increased in ultra-endurance runners; increased at T1 and then decreased at T2 in long distance runners; and decreased at T1 and remained at almost the same level in rugby players. Statistically significant changes were also observed in ox- and red-Cys concentrations within each group at the different times, but showing different kinetic trends (see **Figure 3B2** and **3B3**). No significant differences among the three athlete groups and during the seasonal observations were found regarding total and oxidized cysteinylglycine (ox-CysGly) (**Figure 3C1** and **3C2**). Again, significant seasonal changes were observed for the reduced form (**Figure 3C3**),

with different trends in the groups but quite similar to those observed for the Cys. In contrast, Hcy concentrations (μM) did not change significantly. The values (μM) ranged between 6.46 ± 2.03 (T0) and 5.79 ± 1.69 (T2) in ultra-endurance; 7.90 ± 1.41 (T0) and 8.86 ± 1.68 (T2) in long distance runners; 7.26 ± 1.70 (T0) and 7.14 ± 1.32 (T2) in rugby players.

4. DISCUSSION

The present study was designed to evaluate the oxidative stress plasmatic markers as well as the redox and antioxidant status in high-level players of different sports (i.e., ultra-endurance or long-distance run and rugby) during a competitive season in order to test the effects of training and competition load on oxidative damage and oxy-redox status with the final aim of preventing non-functional overtraining states and optimizing the individual training program. It is well known that overloaded training exerts a significant effect in increasing skeletal muscle damage [6, 7]. High-level performance [8] may be closely relat-

FIGURE 2. Changes of oxidative stress markers in different athlete groups. (A) Protein carbonyls (PC), (B) thiobarbituric acid-reactive substances (TBARS), and (C) total antioxidant capacity (TAC) data were obtained from ultra-endurance (blue) and long-distance (black) runners and rugby players (grey) in the time course of the seasonal competitive period, i.e., at: T0, the beginning; T1, the top; T2, the end. Data are expressed as mean ± SD. Compared to T0, changes over time were significant at: *, $p < 0.05$; #, $p < 0.01$; §, $p < 0.001$; and ¶, $p < 0.0001$.

stress parameters was found, in response to the examined sport activity. Indeed, plasma TBARS and PC reflect the complex peroxidation and carbonylation processes following single or repeated exercises [9–12]. With respect to the basal levels, TBARS and PC were found increased at the end of the competitive periods in all the examined athlete groups (**Figure 2A and 2B**). However, such increases were statistically significant only in the ultra-endurance runners. In rugby players, only the PC levels increased significantly; while an increase was measured in long distance runners, it was not statistically significant. These findings suggested that exercises performed at high-volume and/or endurance levels effectively enhance lipid peroxidation and cause more protein damage. PC was reported to increase following intense training, peaking with overtraining [13]. The rise may be mechanistically attributed to phagocytic cell invasion into the damaged muscle, generating a substantial amount of oxygen radical species, accompanied by underlying inflammation and soreness [14]. At the same time, exercise-induced protein oxidation might also be triggered by iron-containing protein disruption [15, 16]. Indeed the reduction in red blood cell (RBC) levels, ob-

ed to heavy training loads giving rise to a potential basal oxidative stress associated with an adaptation failure in some functions.

The subjects examined in this study represented a homogeneous group of professional players regarding the anthropometric features and the basal levels of the oxidative stress biomarkers. Nevertheless, during the periods of heavy training and competition loads, a different response in the examined oxidative

served during prolonged exercise, could be ascribed to "foot strike hemolysis", that is considered the major cause of hemolysis during running. This process, known to take place owing to the athlete's feet frequently striking on hard surfaces, is a well-known phenomenon mainly in ultra-endurance runners [17]. On the other hand, high TBARS concentration measured in blood is probably ascribable to lipid peroxidation of low-density lipoproteins and oxygen-
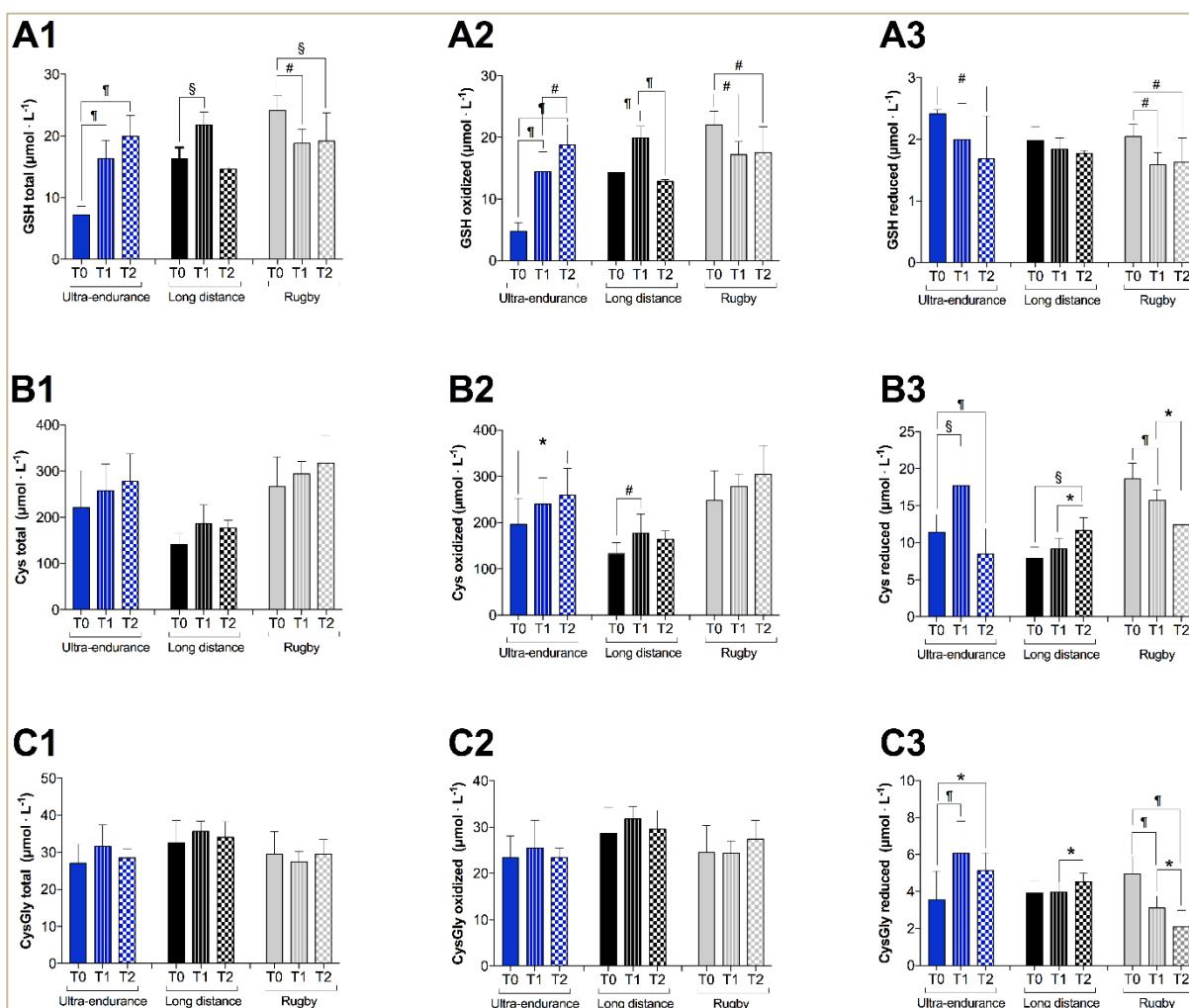
FIGURE 3. Multiple histogram panel plots of the redox status in the plasma of different groups of athletes. Aminothiol concentration data were obtained from ultra-endurance (blue) and long distance (black) runners and rugby players (grey) in the time course of the competitive period, i.e., at: T0, the beginning; T1, the top; T2, the end. Concentrations of total, oxidized and reduced forms of GSH (A1, 2, 3), Cys (B1, 2, 3), and CysGly (C1, 2, 3) are expressed as μM. Data are expressed as mean ± SD. Statistically significant differences (p value across samples by ANOVA) in intra-group comparison are displayed as: *, $p < 0.05$; #, $p < 0.01$; §, $p < 0.001$; and ¶, $p < 0.0001$.

mediated injury of muscle cell membranes [18]. All these changes are associated with overtraining [13].

With regard to the oxy-redox status seasonally monitored in the athletes, TAC activity was not found significantly changed during the monitored time period (**Figure 2C**). This finding seemed to be directly correlated with amino-thiol redox status that was upregulated, as demonstrated by the increase of

oxy and total glutathione and cysteine concentrations particularly measured in ultra-endurance athletes (**Figure 3A** and **3B**). On the other hand, the significant decrease in reduced GSH, Cys, and CysGly concentrations measured in rugby players at T2 (**Figure 3**) suggested that non-enzymatic antioxidants are strongly consumed during the competitive season, and particularly towards the end (T2), and

this fact is closely linked to an overtraining condition. Indeed, during overtraining, GSH supply may not be sufficient to match its enhanced utilization, thereby giving rise to a decrease level in the blood. A GSH clearance increase (e.g., increased consumption by muscle) in the blood might be postulated too since GSH may be used either for ascorbic acid and α-tocopherol regeneration or ROS (i.e., superoxide anion, singlet oxygen) scavenging [19]. Finally, in the present study, in all the categories of athletes and at all sampling times, the Hcy concentration was unchanged and within the normal range. Hcy is a very important parameter to be measured in athletes. In fact, it is well known that high Hcy levels are associated with several disorders such as cardiac, cerebrovascular, and neurodegenerative diseases [20].

5. CONCLUSIONS

Altogether the results achieved in the present study showed that oxidative stress and antioxidant measurements play a peculiar role in monitoring the athletes' training response and performance that was shown to be specifically linked to the played sport activity. Therefore, the seasonal monitoring of athletes may be of great use to plan training load, improve physical performance, and prevent injury. In well-trained professional athletes, only strenuous long duration exercises and exhaustive training will overwhelm the capacity to detoxify ROS, thereby producing significant oxidative stress. Nonetheless, the resulting increased oxidative stress and further cellular susceptibility to damage need more studies in order to evaluate the effects of overtraining and the underlying biochemical mechanisms for ROS production (e.g., iron-containing protein disruption and inflammation).

REFERENCES

1. Mrakic-Sposta S, Gussoni M, Porcelli S, Pugliese L, Pavei G, Bellistri G, et al. Training effects on ROS production determined by electron paramagnetic resonance in master swimmers. *Oxid Med Cell Longev* 2015; 2015:804794. doi: 10.1155/2015/804794.

2. Fry RW, Morton AR, Keast D. Overtraining in athletes: an update. *Sports Med* 1991; 12(1):32–65.

3. Sen CK. Oxidants and antioxidants in exercise. *J Appl Physiol (1985)* 1995; 79(3):675–86. doi: 10.1152/jappl.1995.79.3.675.

4. Jones DP, Carlson JL, Mody VC, Cai J, Lynn MJ, Sternberg P. Redox state of glutathione in human plasma. *Free Radic Biol Med* 2000; 28(4):625–35.

5. Dellanoce C, Cozzi L, Zuddas S, Pratali L, Accinni R. Determination of different forms of aminothiols in red blood cells without washing erythrocytes. *Biomed Chromatogr* 2014; 28(3):327–31. doi: 10.1002/bmc.3056.

6. Kirwan JP, Costill DL, Houmard JA, Mitchell JB, Flynn MG, Fink WJ. Changes in selected blood measures during repeated days of intense training and carbohydrate control. *Int J Sports Med* 1990; 11(5):362–6. doi: 10.1055/s-2007-1024819.

7. Okamura K, Doi T, Hamada K, Sakurai M, Yoshioka Y, Mitsuzono R, et al. Effect of repeated exercise on urinary 8-hydroxy-deoxyguanosine excretion in humans. *Free Radic Res* 1997; 26(6):507–14.

8. Mena P, Maynar M, Gutierrez JM, Maynar J, Timon J, Campillo JE. Erythrocyte free radical scavenger enzymes in bicycle professional racers. Adaptation to training. *Int J Sports Med* 1991; 12(6):563–6. doi: 10.1055/s-2007-1024734.

9. Margaritis I, Tessier F, Richard MJ, Marconnet P. No evidence of oxidative stress after a triathlon race in highly trained competitors. *Int J Sports Med* 1997; 18(3):186–90. doi: 10.1055/s-2007-972617.

10. Alessio HM, Hagerman AE, Fulkerson BK, Ambrose J, Rice RE, Wiley RL. Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. *Med Sci Sports Exerc* 2000; 32(9):1576–81.

11. Metin G, Gumustas MK, Uslu E, Belce A,

Kayserilioglu A. Effect of regular training on plasma thiols, malondialdehyde and carnitine concentrations in young soccer players. *Chin J Physiol* 2003; 46(1):35–9.

12. Knez WL, Jenkins DG, Coombes JS. Oxidative stress in half and full Ironman triathletes. *Med Sci Sports Exerc* 2007; 39(2):283–8. doi: 10.1249/01.mss.0000246999.09718.0c.

13. Margonis K, Fatouros IG, Jamurtas AZ, Nikolaidis MG, Douroudos I, Chatzinikolaou A, et al. Oxidative stress biomarkers responses to physical overtraining: implications for diagnosis. *Free Radic Biol Med* 2007; 43(6):901–10. doi: 10.1016/j.freeradbiomed.2007.05.022.

14. Tiidus PM. Radical species in inflammation and overtraining. *Can J Physiol Pharmacol* 1998; 76(5):533–8.

15. Fatouros IG, Destouni A, Margonis K, Jamurtas AZ, Vrettou C, Kouretas D, et al. Cell-free plasma DNA as a novel marker of aseptic inflammation severity related to exercise overtraining. *Clin Chem* 2006; 52(9):1820–4. doi: 10.1373/clinchem.2006.070417.

16. Jackson MJ. Exercise and oxygen radical production by muscle. In: *Handbook of Oxidants and Antioxidants in Exercise* (CK Sen, L Packer, O Hanninen). Elsevier Science, Amsterdam: Netherlands. 2000, pp. 57–68.

17. Telford RD, Sly GJ, Hahn AG, Cunningham RB, Bryant C, Smith JA. Footstrike is the major cause of hemolysis during running. *J Appl Physiol (1985)* 2003; 94(1):38–42. doi: 10.1152/japplphysiol.00631.2001.

18. Hulbert AJ. On the importance of fatty acid composition of membranes for aging. *J Theor Biol* 2005; 234(2):277–88. doi: 10.1016/j.jtbi.2004.11.024.

19. Sen CK, Packer L. Thiol homeostasis and supplements in physical exercise. *Am J Clin Nutr* 2000; 72(2 Suppl):653S–69S.

20. Herrmann W. The importance of hyperhomocysteinemia as a risk factor for diseases: an overview. *Clin Chem Lab Med* 2001; 39(8):666–74. doi: 10.1515/CCLM.2001.110.