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Original Article

Haematological Parameters and Glutathione Peroxidase Activity in Rugby Players

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ABSTRACT

Background: Rugby is team sport and posses both running and contact play features. In a similar way as another sport producing both physical and psychological stress and both aerobic and anaerobic metabolism, rugby induces intensive oxidative stress. This in turn decreases glutathione peroxidase activity. Furthermore, the complete blood count is severely altered by such a condition. **Methods:** Twelve Italian rugby players were studied. Subjects were studied in the morning of the day of the match and 10 min after the end of match. Erythrocyte glutathione peroxidase activity was analyzed in control and athlete groups pre and post the rugby match both with and without *in vitro* addition of vitamin C. Using a Coulter counter, we measured red blood cell count, hematocrit, hemoglobin, mean corpuscular volume, white blood cell count, platelets, neutrophils, eosinophils and basophils in 12 male athletes, who gave their informed consensus. **Results:** GPX data show that its activity is increased by the *in vitro* addition of Vitamin C (48mM final concentration), both in controls and rugby players. Haematological data describe the major alterations of particulate fraction of blood from rugby players before and after one match and confirm the structural damages in this fraction. GPX activity is worsened by this kind of strenuous exercise and ascorbic acid relieves such damage. Complete blood cell counts of athletes confirm blood cell damages. **Conclusion:** In summary rugby players show evident cell count derangements and water-soluble reducing Vitamin C relieves oxidative damages of samples from test subjects undergone to full match fatigue.

Keywords: Oxidative stress, Sports, Exercise, Rugby, Haematology and glutathione peroxidase.

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INTRODUCTION

Data from literature[1-2] show haematological and biochemical parameter alterations in rugby players after match related to oxidative stress.

Rugby is a model of football that originated from Rugby School in the United Kingdom between 1859 and 1865. It is seen most remarkably in two current sports, Rugby League and Rugby Union, and has determined the evolution of others such as American Football, Canadian football and Australian Rules football. Rugby is a vigorous sport, consisting of both sprinting play and tackling play. Rugby is both an aerobic and anaerobic sport and results indicate significant demands on all energy systems (the relative importance of requiring free oxygen and no-requiring free oxygen energy pathways.) in all playing roles, yet implied a greater reliance on anaerobic glycolytic metabolism, due mainly to their regular involvement in non-sprinting intense activities.[3] Rugby playing appears to impose both psychological and physiological stress on the athletes. In fact, the incidence of injury during a rugby match is high compared to other sports.

Intensive physical aerobic and anaerobic training and competition such as those imposed on professional rugby players can induce an increase of oxidative stress.[4-6]

The total blood count or CBC or haemochrome cytogram (composite greek terms: kromos: color, kytos: cell and metros : measurement, etymologically related to the color and quantity of blood cells) is a complete laboratory examination of blood, which is useful to determine the quantity of blood cells (white blood corpuscles, erythrocytes or red blood corpuscles and blood platelet) and to determine the levels of haematocrit (HcT), and haemoglobin (Hb), an explanatory factor of anemia, and various other blood parameters. Alexander Vastem is largely regarded as being the first researcher to use the total blood count for clinical purposes. Reference ranges used today stem from his clinical trials in the early 1960s.

The figurative elements that circulate in the bloodstream are generally divided into three types: white blood corpuscles, red blood corpuscles, and blood platelet. Irregularly high or low counts may denote the presence of many forms of disorder, and hence blood counts are amongst the most generally performed blood tests in medicine, as they can provide an overview of a patient's general health

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status. A CBC is commonly performed during annual physical studies in some administrations.

GPX, the most powerful antioxidant enzyme (PDB 1GP1, EC 1.11.1.9), is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biological chemistry function of glutathione peroxidase is to reduce lipid hydroperoxides to their homologous alcohols and to reduce free hydrogen peroxide to water. GPX is selenium dependent, the mechanism of GPX involves the selenocystein site, in a E-Se^- form at resting state. This aminoacidic group is oxidized by the peroxide to E-SeOH after entrapped by a GSH molecule to offer Se-SG and by another GSH molecule to Se^- again, liberating a GS-SG by-product.

The GPX reaction is:

$$2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O$$

where GSH represents reduced monomeric glutathione, and GS-SG represents glutathione disulfide. Glutathione reductase (GRD) after reduces the oxidized glutathione to complete the cycle:

$$GS\text{-}SG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+.$$

The human Glutathione Reductase (GRD), also known as GSR, is an enzyme. (E.C. 1.8.1.7) that reduces glutathione disulfide (GSSG) to the sulphydryl form GSH, which is an important cellular antioxidant. For each mole of oxidized glutathione (GSSG) one mole of NADPH is required to produce GSH. NADPH reduces FAD current in GSR to produce a temporary $FADH^-$ anion. This anion then speedily breaks a disulfide bond and leads to Cys^-_{63} nucleophilically attacking the next sulfide group in the GSSG molecule (promoted by His_{467}) thus creating a mixed disulfide bond (GS-Cys_{58}) and a GS^- anion. His_{467} of GSR then protonates the GS^- to form the first GSH. Afterwards, Cys_{63} nucleophilically attacks this sulfide releasing a GS^- anion, thereby creating the second GSH. Therefore, for each GSSG two reduced GSH antioxidant molecules are produced, scavenging reactive oxygen substances in the cell. In cells exposed to high levels of oxidative stress, for example red blood cells (RBC), till 10% of the glucose expenditure may be directed to the pentose phosphate pathway (PPP) for the production of the NADPH needed for this response. In erythrocytes, if the PPP does not function, then the (RBC) oxidative stress will lead to lysis, anemia.

This scavenging activity was studied after the "*in vitro*" treatment with Vitamin C.

Vitamin C or L-ascorbic acid or L-ascorbate is an basic nutrient for humans and secure other animal species. In living organisms, ascorbate reacts as an antioxidant by protecting the organism against oxidative stress.[7] It is still a cofactor in at least eight enzymatic reactions including several collagen synthesis reactions that cause the most severe manifestations of scurvy when they are dysfunctional. Food Standards Agency (UK), 2007).

In animals these reactions are particularly important in wound-healing and in preventing bleeding from capillaries.

Vitamin C is water-soluble and is maybe the most important antioxidant in extracellular fluids, but is also effective in cytosol.[8-9] Vitamin C is richer in tissues in which ROS production is more important. This phenomenon is defined as an adaptation against oxidative stress.[9] Thus, Vitamin C supplementation has often been studied. In athletes, its preventive effects against oxidative stress are debated.[10-13]

A deficiency in Vitamin C has unfavorable effects on lending and Vitamin C supplementation (particularly in combination with other antioxidants such as Vitamin E) helps to maintain a sufficient Vitamin C level in tissues.[14]

The objective of this work is to test the effect of training and competition charge oxidative stress, haematological and biochemical (GPX) parameters in rugby players during a competitive season.

METHODS

Subjects and Study Protocol

The subjects were 12 Italian rugby players who belonged to Italian rugby teams (National Rugby Championship, that is the Italian Championship group C South.). All subjects played rugby for 80 min with an interval of 10 min. Subjects were studied in the morning of the day of the match and 10 min after the end of match. Both haematological and biochemical (GPX) parameters were examined.

Approval was obtained from the Ethics Committee (SRPL/CLI/10-11/001). Prior to the study Informed consent was obtained from all the study participants. The study was conducted according to the criteria set by the *Declaration of Helsinki*.

Blood Biochemistry

Exercised athlete blood samples (5ml) were collected 10 min before exercise, in vacutainers containing 0.15% EDTA and refrigerated at 8°C and after 5 min recovery, post-exercise periods. Each 10 ml. blood sample was taken from the forearm vein of seated subjects. Athlete samples instead were drawn similarly, before warming up by jogging. The venipuncture was performed by an official physician. Samples were drawn into an evacuated, heparinized blood collection tube for chemical clinical analyses, and 3mL into an evacuated ethylenediaminetetraacetic acid tube for GPX analyses. All samples were refrigerated at 8°C, then transformed with Drabkin's reactant (from Sclavo diagnostics, Siena, Italy) before enzyme activity determination.[15]

The RBC (red blood cells) GPX activity was measured. Biochemical parameters were glutathione peroxidase (GPX). The studied scavenger activity of the ROS is that of the multienzymatic complex GPX-GRD which develops a greater disposal of the ROS from the systemic circulation. GPX activity is determined according to Paglia and Valentine[16] as modified by Gallo *et al*. 2009[17] in RBC samples from athletes both before and after competition (rugby). Blood, 100µL of the previous samples, is centrifuged (2000 rpm/min) and washed within three hours[18] twice with 5 mL of 0,9% NaCl. Isolated RBCs are haemolyzed by addition of 1 mL of distilled H_20. Haemoglobin concentration is determined by mixing 1mL of Haemoglobin test (Sclavo diagnostics, Siena, Italy) with 0,1 mL of haemolysate. The absorbance at 546 nm is measured in a Shimadzu UVPC 2100 spectrophotometer (A546 x 16 = mg Hb/mL) against a blank containing water instead of haemolysate. The haemolysate is exactly diluted to 3 mg of Hb per millilitre. From this solution, 1 mL is mixed with 0.5 mL of conversion solution (4.5 mM KCN and 0.45 mM $K_3[Fe(CN)_6]$ regulated with 0.25 M potassium dihydrogen phosphate to pH 7.0). After 5 min, conversion to cyanmethemoglobin is complete at ambience temperature between brackets. GPX activity was evaluated by a modified method of Paglia and Valentine,[16] similar to Gallo and Martino.[17] The activity was thus evaluated spectrophotometrically by connection the oxidation of glutathione and NADPH using GRD. Briefly, 1mL of test mixture contains enhance concentrations of the following chemicals: 0.25M K_2PO_4 (pH 7.0), 2,5mM EDTA, 6 U/mL GRD, 15mM glutathione and 1,5 mM reduced NADPH and tissue excerpt (0,5mL) was added in the spectrophotometer cuvette along with 0,1mL of 12 mM cumene hydroperoxide, a appropriate substance for GPX.

The kinetics are acquired over a 120 sec. period by an UV2100 Shimadzu spectrophotometer at 366 nm and 37°C, Vmax and Km values were determined by Michaelis and Menten graphical method by GraphPad Prism® 5.02 program and the statistical significance was evaluated by T-Student.[19] The regression analysis is performed by SPSS® 17.0.2

program by one way ANOVA,[20-21] according to the least squares method. The reagents were all analytical grade from Sigma (St. Louis USA).

T. ferdinandiana is a widespread species in the northern part of Australia, and is particularly widespread in the tropical forests of the Kimberley, up to Arnhem Land, in the east (Figure 1).

It has a high concentration of Vitamin C in its fruits: the recorded concentrations of 2,300–3,150 mg / 100 g wet weight with peaks up to 5,300 mg / 100 g, compared to 50 mg / 100 g for oranges, place it among the major natural sources of this Vitamin.

Serum ascorbate concentrations were determined according to Moeslinger *et al*. (1994; 1995).[22-23]

Reagents: Ascorbic acid, ascorbic acid oxidase (EC 1.10.3.3), citric acid 1-hydrate, monosodium phosphate, dithiothreitol, and desferrioxamine were obtained from Sigma Chemical Co., St. Louis, MO. Methanol (HPLCgrade) was supplied by Mallinckrodt Chemicals, Paris, KY. Chelex 100 (200-400 mesh) was buyed from Bio-Rad Labs., Richmond, CA. Chelexed buffers were prepared by stirring 100 mL of buffer with 10 g of Chelex resin for 60 mm at room temperature to remove trace metal residues, then filtering the buffer from the resin.

Spectrophotometry: Spectrophotometric measurements were taken with a Shimadzu 4A chromatograph equipment with SPD-6A UV/VIS spetrophotometric detector with a thermostating system. We used 1-cm pathlength cuvettes and a sample volume of 1 mL.

In these experiments, carried out on samples of hemolyzed human blood was used (Hb =3mg/ml) in normal medium of incubation containing GSSG 15 mM and ascorbic acid (48μM).

Each 10 ml blood sample was taken from the forearm vein of seated subjects. Each kind of normal blood cell are measured using an automatic cell counter.

Blood samples were gathered from the antecubital vein after overnight fasting. No unscheduled, intensive training was done the night before the blood sampling. Each 10-ml blood sample was collected in an uncoated vacuum tube and immediately centrifuged for biochemical analyses and a 2-ml aliquot was gathered in a tube coated with potassium EDTA for the total blood count. The total blood count included white blood corpuscles (WBC), red blood corpuscles count (RBC), hematocrit (Hct), and hemoglobin (Hb). The counting was made using a Coulter-STKA auto-analyzer.

Figure 1: Kakadu, also known as gubinge or murunga (Terminalia ferdinandiana *Exell*) is an arboreal species of the Combretaceae family.

The Coulter-STKA is a fully automatic system for haematological tests. A Coulter counter is an apparatus for counting and sizing particles and cells. It is used, for example, for bacteria or prokaryotic cells and air quality particle size distributions. The counter detects alteration in electrical conductance of a short aperture as fluid containing cells is drawn through. Cells, being non-conducting particles, change the effective cross-section of the conductive channel.

It was an American creator named Wallace H. Coulter, who first projected the theory behind its operation in 1947 while experimenting with electronics. Coulter established that electrical charge could be used to define the size and number of microscopic particles in a solution. This phenomenon is now notorious as the Coulter Principle.

Its primary function being the quick and accurate test of total blood counts (often referred to as CBC). The CBC is used to evaluate the number or proportion of white and red blood cells in the body.

Statistical analysis

The results of each measurement are presented as mean (±SD). Data of blood biochemistry at both time points (before and after) were compared by one-way ANOVA. Differences between control and pre and post exercise data were evaluated and considered to be significant at $p<0.05$ level.

RESULTS

Figure 2 shows a typical GPX time course in control sample. Figure 3 shows GPX time course in presence of L-ascorbic acid in the same control sample to evidence its role as antioxidant. In Figure 4 are represented time courses pre and post exercise in presence or absence of vitamin C and GSH 15mM. The data on GPX activity make evident an activity decrease in RBC membranes. After the increase of oxidative stress, indeed, the GPX activity damage is evident after competition. The haematological data were determined on the sap of athletes more/less to exercise.

Table 1 shows the data of enumerations of blood morphologic elements before and after agonist activity. The parameters WBC and neutrophils have an enhancement after sport activity. Platelets have a slight increase. Table 1 sets out the parameters for the haemoglobin (Hb), haematocrit (Hct) and counts of red blood corpuscles (RBC), respectively. From the examination of the described tables follows a slight but not significant variance of HB blood content and Hct parameter.

In summary Vitamin C increases GPX reducing activity in control samples. The addition of Vitamin C increases GPX activity in both pre and post exercise groups of RBC.

CBCs on blood of either pre and post exercise subjects show statistically significant variation of white blood corpuscles, blood platelet and Neutrophils %.

Samples from trained athletes demonstrate significant variations of haematological parameters in comparison with the data of sedentary athletes. Only platelets data from post match athletes are significantly inferior than control data. GPX data in post match athletes are significantly lower than control ones. This demonstrates that trained athletes show White blood corpuscles, Blood platelet and Neutrophils % significantly affected by frequent rugby activity and their parameters are stable even post match. GPX instead is seriously affected only by match stress.

DISCUSSION

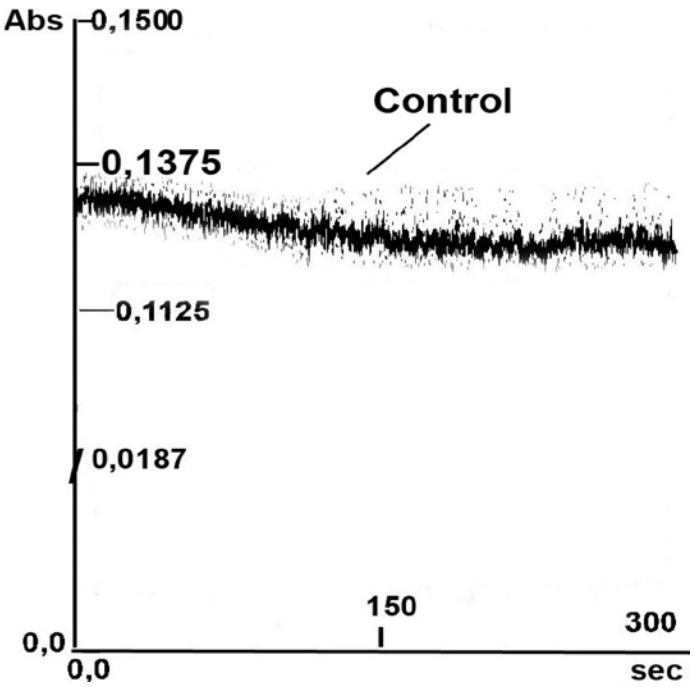

Figure 2: Time course of spontaneous GPX reaction in human blood reference samples 300 sec.

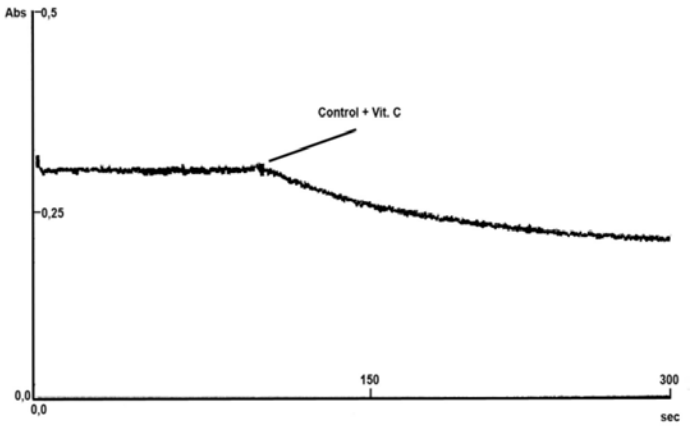

Figure 3: Typical time course with Vitamin C addition 48µM to start the reaction.

To better define the effect of exercise on ROS scavenging activities the model under investigation is trained human athletes, such as rugby players. Rugby is both an aerobic and anaerobic vigorous sport, as literature results[1] indicate significative requests on all energy systems in all playing roles. In this instance the authors study the GPX activity and total blood count (CBC) of control (trained players before the match at rest) and exercised 12 players of an Italian team (National Rugby Championship, that is the Italian Championship group C South). Studies are performed at mid-season in April and May (second round of the championship).

GPX activities were demonstrated also after *in vitro* addition of L-ascorbate (48 µM final concentration) to sampling buffer. In trained rugby players RBC GPX optimized activity is significantly diminished after the match.

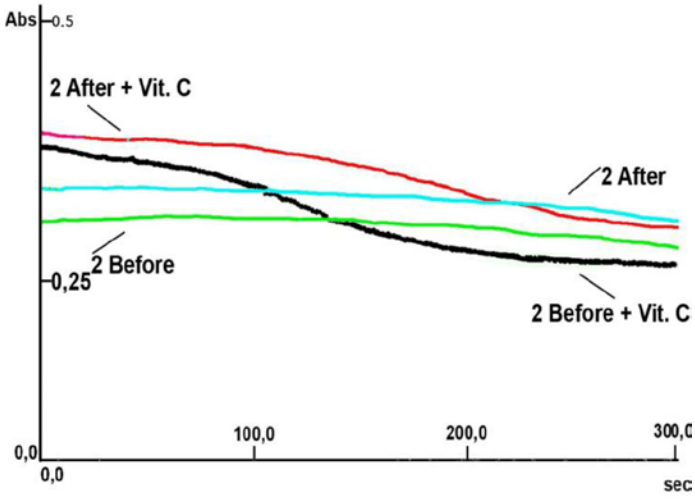

Figure 4: Time course of GPX activity measured in sample N°2 of rugby players: Before match, After match, After match + Vit. C 48µM, 2 Before match + Vit. C 48µM.

Table 1: Main blood parameters before and after competition.

Parameters	Controls (*n*=12)	Rugby players (*n*=12)		*P*
		Before	After	
RBC (10^6/µL)	5.26	4.81	4.94	n.s.
Hb (g/dL)	17.0	15.4	15.8	n.s.
Hct (% volume)	46.4	42.2	43.4	n.s.
MCV (µL)	88	84	88	n.s.
WBC (10^3/µL)	6.3	5.1*	6.8	* $p<0.05$
PLT (10^3/µL)	328	269*	274*	* $p<0.05$
Neutrophils (%)	55	42*	54	* $p<0.05$
Eosinophils (%)	0	1	0	n.s.
Basophils (%)	<1	<1	<1	n.s.
GPX (µM/min/mg)	0.0250	0.0213	0.0085**	** $p<0.005$

CBC haematological parameters are studied by Coulter Counter and demonstrate some significative changed (WBC, PLT and neutrophils %) in "after match" samples.

Vitamin C *in vitro* treatment of RBC preparations increases GPX activity in control, before and after match samples.

Our data are well in accordance with results of Finaud *et al.* (2006)[1] on full season [that is severe phases of training and competition (T1 and T4), the lower intense period of training (T2), compared to the control time (T3).]: He states "In December (T1), during the beginning of the competitive period of the season. In April (T2), during the most important period of competition. In September (T3), at the opening of the season. In December (T4), during the beginning of the competitive period of the season." Oxidative stress enzyme data of rugby players under the action of the reducing agent Vitamin E (only in T1 (-8.7%). Only T2 was followed by less neutrophils (-8.5%). The daily mean value of ascorbic acid administration *in vivo* is close to the dose added *in vitro* to isolated RBC preparations.[1]

Few studies are available on the more apposite resting methods to present effective recovery from a match with or without loaded exercise. Fifteen Japanese college rugby players were studied. Between them seven executed only normal daily activities and eight performed additional less intensity exercise during the after-match rest phase. Players were examined just before and instantly after the match (as described in our present research) and one and two days after the match. Data from Table 1 of Suzuki *et al.* (2004)[2] show variations in total white blood corpuscle and neutrophil counts post the rugby match. In both groups, total white blood corpuscle count was remarkably increased instantly after the match and so was the neutrophil count ($p<0.01$). There were no variations in neutrophil counts between the two groups at any period points.[2]

According to Tauler *et al.* (2008),[24] the ANOVA analysis of the plasmatic antioxidant levels pre and post the football match performed after supplementation of reducing agents induced higher neutrophil counts. The circulating amount of neutrophils increased by approximately 67% in the placebo unit and about 85% in the supplemented one. The game influenced the neutrophil activity of glutathione peroxidase. Glutathione peroxidase activity decreased significantly in both placebo and supplemented units.[24]

Authors[25] summarize the pre-(At Rest) and post exercise erythrocyte GPX activity of three groups (sedentary controls, trained rugby players and weekend warriors). The trained group shows almost stable GPX activity and the "weekend warriors" group had the highest post exercise RBC GPX activity in comparison to the other groups. This GPX activity before exercise was similar in all groups.[25]

Schippinger *et al.* (2002)[26] compared the serum concentrations of both ascorbate and fat-soluble vitamins in the eight subjects (Professional American football players). During competition: at timepoints no. 1, (precompetition season; March), no. 3 (June), and no. 4 (at the finish of season; July). Serum ascorbate concentrations were in the regular range (69·5 ± 16·6 μM)[27] with a tendency towards lower values at time-point no. 3. Ascorbate serum concentrations instead increased almost significantly between time-point no. 3 and no. 4 (paired *t*-test, $P= 0.04$).[26]

Significant differences were observed for haemoglobin (Hb), erythrocytes, haematocrit (Hct), and mean corpuscular volume (MCV).[28]

The cell component of immune system is modified by inflammation. This can be started by exercise-induced tissue damages and alterations which can be quantified by the measurements of GPX activity. Indeed, high intensity exercise results in neutrophils and monocytes, but extensively all White blood cells, increase subsequently resulting in an oxidative stress. Probably this rebound can be described by body dehydration following the after-match physical stress.

The RBC membrane GPX activity worsened after rugby match, due to lipid peroxidation (oxidative damage) as described by Finaud *et al.* (2006).[1] This is the reason why ascorbic acid *in vitro* treatment (48 mM final concentration) was performed to evaluate the partial recovery of scavenging enzymatic activity.

CONCLUSION

Comprehensively described results and those from other authors on oxidative stress stimulated by exercise in during a rugby game/match show erythrocytes membrane GPX activity decrease. CBC data demonstrate that RBC counts and correlated parameters are almost invariant, however WBC and platelet counts significantly increase as exercise could induce inflammations (increased Neutrophil and Monocyte counts). After exercise dehydration of athletes partially contributes to a limited rebound of haematological parameters.[29]

In vitro therapy with 48 μM ascorbic acid f.c. partially recovers oxidative damage on GPX activity of RBC membranes from exercised athletes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ABBREVIATIONS

HcT: Haematocrit; **Hb:** Haemoglobin; **GPX:** Glutathione peroxidase; **GRD:** Glutathione Reductase; **CBC:** Total Blood Count; **RBC:** Red Blood Cells; **Vitamin C;** L-ascorbic acid or L-ascorbate; **WBC:** White blood corpuscles; **MCV:** Mean Corpuscular Volume.

SUMMARY

Rugby is a model of football. Rugby is a vigorous sport, consisting of both sprinting play and tackling play. Rugby is both an aerobic and anaerobic sport. Vitamin C increases GPX reducing activity in control samples. The addition of Vitamin C increases GPX activity in both pre and post exercise groups of RBC. CBCs on blood of either pre and post exercise subjects show statistically significant variation of white blood corpuscles, blood platelet and Neutrophils %.

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