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Research article

Red blood cell and whole blood glutathione redox status in endurance-trained men following a ski marathon

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Abstract
The aim of the present study was to evaluate the changes in glutathione redox ratio ($GSSG \cdot GSH^{-1}$) in red blood cells (RBCs) and whole blood in well-trained men following a ski marathon. 16 male subjects (27.0 ± 4.7 yrs, 1.81 ± 0.06 m, 77.6 ± 9.6 kg, VO_2max 66.2 ± 5.7 $ml \cdot kg^{-1} \cdot min^{-1}$) were examined before the competition (pre-COMP), after the competition (post-COMP) and during an 18-hour recovery period (RECOV). There was a slight decrease in reduced glutathione (GSH) in blood and in RBCs in post-COMP. During RECOV, the GSH level in blood was reduced, the GSH level in RBCs was significantly elevated (a statistically significant difference as compared to the pre-COMP level). The post-COMP $GSSG \cdot GSH^{-1}$ in full blood did not increase significantly, but its increase was statistically significant during the 18-hour recovery period. During the post-COMP and RECOV, the $GSSG \cdot GSH^{-1}$ in RBCs slightly decreased in comparison with the pre-COMP. Vitamin C concentration in serum increased in post-COMP (49% vs. pre-COMP) and decreased to the baseline level during RECOV. In conclusion, our data show that acute exercise slightly increases the $GSSG \cdot GSH^{-1}$ in whole blood, while $GSSG \cdot GSH^{-1}$ in RBCs significantly decreases. Thus, exercise-related changes in the non-enzymatic components of the glutathione system (GSSG and GSH) in whole blood and RBCs are not identical.

Key words: Free radicals, antioxidants, glutathione, vitamin C, exercise.

Introduction

Different kinds of tightly associated reactions occur in the human organism, the purpose of which is to guarantee homeostasis. Reactive species (including free radicals) participate in some physiological oxidative reactions. However, when these reactions cross threshold levels, damaging factors will prevail and lead to oxidative stress (OxS) (Finaud et al., 2006; Halliwell, 2001).

It is well known that intensive exercise is related to increased generation of reactive oxygen species (ROS), which results in OxS (Finaud et al., 2006; Khanna et al., 1999; Maughan and Gleeson, 2004; Oztasan et al., 2004; Ramel et al., 2004; Urso and Clarkson, 2003). ROS mainly results from damaged mitochondria of the muscles, but it is also produced by red blood cells (RBCs) (Clemens and Waller, 1987; Turrens, 2003). In order to prevent OxS, there is an elaborate antioxidant defence system consisting of enzymatic antioxidants, such as catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and numerous non-enzymatic antioxidants, including glutathione, vitamin C, E, Q, carotenoids, and uric acid (Tauler et al., 2003; Urso and Clarkson, 2003). Thus, it is important that the antioxidant defence system in blood, especially in RBCs, is effective and recovers properly after exhaustive physical load.

Recent research has shown that after intensive training, the level of antioxidants decreases and lipid peroxidation in blood and in other tissues increases, and the ROS production is also elevated in RBCs (Cazzola et al., 2003; Tauler et al., 2003). It is notable that the damage of RBCs by ROS may become evident due to limited antioxidant defence systems mainly during the early post-exercise period (Marzatico et al., 1997). At the same time, the intensity of oxygen consumption and the status of the cellular antioxidant mechanism are associated with the quantity of oxidative damage during the exercise and recovery period (Cazzola et al., 2003; Evans, 2000; Maughan and Gleeson, 2004).

The principal cellular non-enzymatic antioxidant system is the glutathione system (Halliwell, 2001). Increased oxidation of reduced glutathione (GSH) during physical exercise has been shown in research. At the same time, the post-exercise level of GSH did not increase (Viguie et al., 1993). The homeostasis of the glutathione system is guaranteed by the GSH storages in the liver. However, long-term exercise may lead to a decreased GSH level in the liver (Ji, 1999) and consequently to disturbances of glutathione redox mechanisms. The deficiency of GSH is associated with an increase in glutathione redox ratio ($GSSG \cdot GSH^{-1}$), and elevated lipid peroxidation in skeletal muscles as well as in heart muscles (Ji, 1999). In humans, the highest levels of GSH are found in RBCs, while the concentration of GSH in plasma is substantially lower (4-6 µM) (Zilmer et al., 2005). Thus, it is very important to examine how these exercise-induced changes are accounted for by the RBCs (where the glutathione concentration is high) or by blood plasma. Only testing the whole blood glutathione levels may not adequately reflect the actual target of exercise-induced influences. In addition, glutathione and vitamin C work closely together in human body cells – both are needed for conversion of the radical form of vitamin E back to non-radical.

Thus, the purpose of the present study was to evaluate the changes in glutathione redox ratio ($GSSG \cdot GSH^{-1}$) in RBCs and whole blood in well-trained men during a ski marathon.

Methods

Subjects

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Sixteen voluntary endurance-trained male subjects who participated in a ski marathon in Estonia (Haanja, 40 km distance, classic style) were examined. Forty-eight hours before the competition, the subjects passed a physical examination and a maximal oxygen consumption (VO_2max) test. No participants showed any signs of bacterial or viral symptoms. The study protocol was approved by the Ethics Committee, University of Tartu. Informed consent was obtained from each participant.

Maximal oxygen consumption (VO_2max)

The subjects underwent a maximal exercise test to determine maximal O_2 consumption. All subjects performed an incremental test on a treadmill (Runrace HC 1400, Technogym, Gambettola, Italy) using a standard protocol test. Expired gas was analyzed continuously using an online system (TrueMax 2400, ParvoMedics, East Sandy, Utah, USA). The subjects were required to meet two of three standard criteria for having achieved VO_2max (heart rate ≥ age-predicted maximum heart rate, respiratory exchange ratio ≥ 1.10, rating of perceived exertion ≥ 19) (6-20 points, 19 is equal to 100% effort or extremely hard; 20 points is equal to exhaustion) (Davis, 2006). The exercise tests were carried out 2-4 h after breakfast.

Anthropometric measurements

The subjects' height and weight were determined by the Martin metal anthropometer (±0.1 cm) and clinical scales (±0.05 kg), respectively. The body mass index (BMI) was calculated ($kg \cdot m^{-2}$). The near infrared interactance method (Futrex-A/WL 5000, USA) was used to estimate body fat percentage.

Laboratory procedures

Venous blood samples were drawn from the antecubital vein. The pre-competition level samples (pre-COMP) were obtained two days prior to the competition. Samples were also taken immediately after the competition (post-COMP), and after an 18-hour recovery period (RECOV).

Haemoglobin and haematocrit were estimated by the Ssmex XE 2100 autoanalyser (Sysmex Corporation, Japan). The values obtained were used to calculate changes in plasma and blood volume (Dill and Costill, 1974).

Vitamin C was analyzed in serum using the automatic oxidation method of Tulley with the use of the free radical of 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy and o-phenylenediamine (Ihara et al., 2000).

Oxidized and reduced glutathione. In order to measure whole blood glutathione after drawing a blood sample, 500 µL of whole blood was immediately transferred into a tube containing metaphosphoric acid. The solution was mixed and centrifuged (4000 rpm, 4ºC, 10 minutes), the supernatant was collected. For the measurement of glutathione in erythrocytes, the whole blood was centrifuged for 10 minutes at 3000 rpm and the plasma was aspirated. Then the equal volume of the 10% solution of metaphosphoric acid and the precipitate was mixed and kept at room temperature for 10 min. The sample was centrifuged at 7000 rpm for 10 min and the supernatant was collected. Samples for reduced and oxidized glutathione (GSH, GSSG, respectively) were stored at –70ºC until the analysis. Total glutathione (tGSH) and GSSG were measured by the enzymatic method of Tietze (1969), which was modified and described by Kullisaar et al. (2003). The content of GSH was calculated as the difference between tGSH and GSSG. The glutathione system redox potency was expressed as the glutathione redox ratio ($GSSG \cdot GSH^{-1}$).

Dietary intake

All subjects assessed their usual dietary habits three days before the competition (in addition to breakfast on the competition day). The scales were used (±2.0 g) for quantified records. All food items consumed were transformed into nutrients using the adapted MicroNutrica program version 2.0 (Finland). The following food characteristics were used: total energy intake of carbohydrates, fats and proteins, vitamin C, E, A, and B-group intake.

Statistical analysis

The results are presented as a mean ± standard deviation. All the data were tested for their normal distribution. ANOVA for repeated measures was used to determine the significance of the differences in parameters measured in pre-COMP, post-COMP, and RECOV. When significant ANOVA was found, the paired *t*-test for dependent data was used. Pearson correlation coefficients (r) were used to evaluate associations between different variables of interest. Calculations were performed with the SPSS, Version 11.0 (SSPS Inc, Chicago, IL) statistical package. Statistical significance was defined as $p<0.05$.

Table 1. Mean anthropometric, training, and aerobic capacity characteristics of the subjects. Values are means (± SD).

Parameter	Subjects (n=16)
Age (yrs)	27.0 (4.7)
Height (m)	1.81 (.06)
Weight (kg)	77.6 (9.6)
BMI ($kg \cdot m^{-2}$)	23.7 (1.9)
Fat percentage (%)	14.3 (4.2)
Years of training (yrs)	10.1 (5.3)
Training ($h \cdot week^{-1}$)	8.6 (2.2)
VO_2max ($l \cdot min^{-1}$)	5.1 (0.7)
VO_2max/kg ($ml \cdot kg^{-1} \cdot min^{-1}$)	66.2 (5.7)
Maximal HR ($beats \cdot min^{-1}$)	193.0 (9.7)
Mean competition time (minutes)	179 (41)
Mean HR during the marathon ($beats \cdot min^{-1}$)	169.3 (9.8)
% of the maxHR during the marathon	87.8 (4.7)

Note. BMI- body mass index; VO_2max – maximal oxygen consumption; HR – heart rate

Results

Table 1 provides descriptive information about mean age, anthropometric and physical working capacity data (VO_2max, $VO_2max \cdot kg^{-1}$) as well as individual maximal heart rate (HR), mean HR and percentage of the maximal HR during the ski marathon. The selected nutritional characteristics of the subjects are presented in Table 2. Daily nutritional intake of vitamin C varied individually – from 40.4 mg to 340.1 mg. Table 3 shows the changes in haemoglobin, haematocrit, plasma volume, GSSG, GSH and tGSH in whole blood and RBCs following the competition. There was a slight decrease in reduced glu-

tathione in whole blood as well as in RBCs immediately after the competition (statistically non-significant). The recovery period data demonstrate that the GSH level in whole blood was continuously reduced (a statistically significant difference in comparison with pre-COMP level). At the same time, the reduced glutathione level in RBCs (eGSH) was significantly elevated (a statistically significant difference as compared to pre-COMP level).

Table 2. Nutritional intake of the subjects. Values are means (±SD).

Total energy intake (kcal)	3213.2 (961.9)
Protein (g)	109.4 (30.0)
Fat (g)	130.5 (51.1)
Carbohydrate (g)	372.5 (110.2)
Protein (% energy)	14.1 (1.9)
Fat (% energy)	35.9 (6.5)
Carbohydrate (% energy)	47.5 (6.9)
Vitamin C (mg)	118.4 (89.4)
Vitamin E (mg)	14.9 (7.2)
Vitamin A (µg, RE)	2112 (2816)

The mean values of $GSSG{\cdot}GSH^{-1}$ in whole blood and in RBCs are presented in Figure 1. The post-COMP $GSSG{\cdot}GSH^{-1}$ in whole blood did not significantly increase, but the increase was statistically significant (from 0.08 up to 0.11, in pre-COMP *vs.* RECOV, respectively) during the 18-hour recovery period. In RBCs, $GSSG{\cdot}GSH^{-1}$ baseline value (pre-COMP) was lower than in whole blood (statistically non-significant). The post-COMP data did not show significant changes in comparison with pre-COMP values. Mean $GSSG{\cdot}GSH^{-1}$ in RBCs slightly decreased in the recovery period in comparison with the pre-competition level.

Mean vitamin C concentration in serum significantly increased after the ski marathon (post-COMP) (49% in comparison with pre-COMP) and practically decreased to the baseline level during the recovery period (RECOV) (Figure 1). The change in vitamin C concentration ($C_{change1}$= vitamin C during post-COMP – vitamin C pre-COMP) varied individually (-5.7 $mg{\cdot}L^{-1}$ to 12.1 $mg{\cdot}L^{-1}$, mean value 3.1 ± 5.1 $mg{\cdot}L^{-1}$). Nutritional intake of vitamin C did not show statistically significant relationships between vitamin C concentrations in the serum.

An inverse association was found between mean HR during the marathon and serum $C_{change1}$ ($r = -0.670$; $p = 0.006$). Relative HR (% of the maxHR during the marathon, Table 1) also showed a statistically significant inverse association with $C_{change1}$ ($r = -0.563$; $p = 0.029$). There were no statistically significant associations between nutritional intake of vitamin C (mg, $mg{\cdot}kg^{-1}$), ($C_{dietary+\ suppl}$, $mg{\cdot}kg^{-1}$) and glutathione redox ratio in whole blood and in RBCs.

Discussion

It is well known that glutathione is a crucial cellular multivalent bioprotector playing a role in a number of processes as the regulation of the levels of reactive species (also known as proinflammatory factors), maintenance of redox potential, and transport of amino acids (Halliwell, 2001; Meister, 1989; Zilmer et al., 2005). Moreover, a recent adapted conception of OxS is advanced as "a disruption of redox signalling and control" (Jones, 2006; Sies and Jones, 2007). This emphasizes an impact of glutathione and its redox ratio as good tools for the quantification of OxS and signalling the regulative role of GSH (Karelson et al., 2002; Zilmer et al., 2005). The present study evaluates the changes in glutathione redox ratio expressed as $GSSG{\cdot}GSH^{-1}$ in RBCs and whole blood in well-trained endurance athletes following a ski marathon. Acute exercise slightly increased the $GSSG{\cdot}GSH^{-1}$ in whole blood, while $GSSG{\cdot}GSH^{-1}$ redox status decreased in RBCs.

The glutathione redox ratio ($GSSG{\cdot}GSH^{-1}$) is an important cellular oxidative stress marker, which stays below 0.1 under normal physiological conditions (Wu et al., 2004). During physical load, the oxidation of GSH increases; during the recovery period, oxidized GSH level comes down to its baseline level. The lower level of GSH is related to different physiological and biochemical disturbances during long-lasting physical exercise – the secretion of GSH from the liver to plasma is elevated, which must ensure the homeostasis of the blood (Ji, 1999). However, the acute exercise-induced effect on the GSH is not well established (Viguie et al., 1993). It has been shown that only exhaustive exercise induces glutathione oxidation (Sastre et al., 1992). Thus, the increase

Table 3. Changes in haemoglobin, haematocrit, plasma volume, oxidized, reduced and total glutathione in blood and red blood cells (RBCs). Values are means (±SD).

Parameter	Pre-COMP	Post-COMP	RECOV
Hgb ($g{\cdot}L^{-1}$)	139.4 (8.5)	137.9 (10.0)	130.9 (7.1)*** ###
Hct (%)	42.3 (1.6)	41.7 (2.3)	40.1 (1.8)*###
Plasma volume change (%)	-	-2.6 (6.8)	10.2 (5.1)
GSSG ($\mu M{\cdot}L^{-1}$)	89.5 (19.6)	94.6 (21.1)	99.3 (34.2)
GSH ($\mu M{\cdot}L^{-1}$)	1120 (129)	1114 (145)	1029 (153)*
tGSH ($\mu M{\cdot}L^{-1}$)	1209 (128)	1209 (153)	1129 (150)*
eGSSG ($\mu M{\cdot}g^{-1}$ Hb)	1.07 (0.26)	0.96 (0.15)	0.96 (0.25)
eGSH ($\mu M{\cdot}g^{-1}$ Hb)	10.6 (2.1)	10.0 (1.9)	11.4 (1.6)*
etGSH ($\mu M{\cdot}g^{-1}$ Hb)	11.5 (1.8)	11.0 (1.9)	9.6 (2.5)**

Note. Hgb − haemoglobin; Hct − haematocrit; GSSG − oxidized glutathione in blood; GSH − reduced glutathione in blood; tGSH − total glutathione in blood; eGSSG − oxidized glutathione in RBCs; eGSH − reduced glutathione in RBCs; etGSH − total glutathione in RBCs; pre-COMP − precompetition level; post-COMP − immediately after the competition; RECOV − 18-hour recovery period;
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, different from pre-COMP.
$p < 0.001$, different from post-COMP.

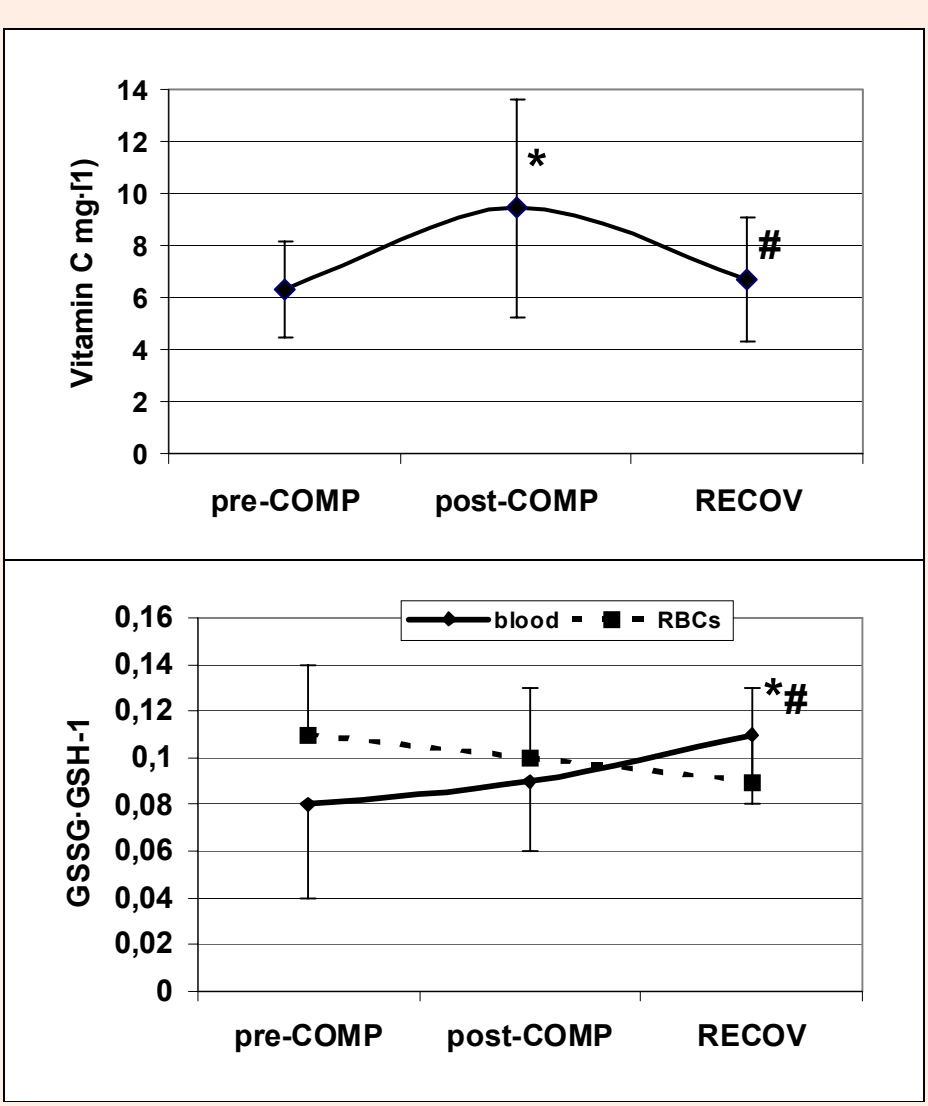

Figure 1. Vitamin C in serum, GSSG·GSH^{-1} ratio in blood and in RBCs during the ski marathon (x±SD).
Note. pre-COMP − precompetition level; post-COMP − immediately after the competition; RECOV − 18-hour recovery period; * $p < 0.05$, different from pre-COMP; # $p < 0.05$, different from post-COMP

in GSSG·GSH^{-1} may be influenced by several factors, including the intensity, mode and duration of exercise as well as the aerobic fitness of the subjects.

In our study, whole blood glutathione redox ratio (GSSG·GSH^{-1}) slightly increased after the ski marathon (Figure 1). During the recovery period, the whole blood GSSG/GSH ratio was significantly higher (up to 0.11) as compared to the pre-COMP and post-COMP level. Under the same procedure (post-COMP and RECOV), the GSSG·GSH^{-1} in RBCs decreased in comparison with pre-COMP. The present data reveal that exercise-induced glutathione redox status (GSSG·GSH^{-1}) did not remarkably exceed the respective threshold value. As seen in Table 3, the elevation of GSSG·GSH^{-1} in blood seem to be accounted for by the decreased production of reduced glutathione. On the other hand, the oxidized glutathione was also elevated. However, the increase was not statistically significant. In RBCs, glutathione redox ratio (GSSG·GSH^{-1}) slightly decreased in post-COMP as well as RECOV period as compared to the pre-COMP level (a statistically non-significant decrease). Reduced glutathione (eGSH) in RBCs was remarkably elevated during the recovery period. Thus, exercise-induced changes in the glutathione system seem to be effective in RBCs and may prevent the damage from the ROS.

It is known that exercise-induced OxS elevates serum vitamin C level, which guarantees the homeostasis of an organism, especially in the RBCs (Viguie et al., 1993; Tauler et al., 2003). It has been suggested that an exercise-induced increase in vitamin C is associated with the exercise-related increase in cortisol concentration, which promotes the release of ascorbic acid from adrenal glands and the mobilization of ascorbic acid from other tissues (Gleeson et al., 1987, Viguie et al., 1993). Dietary supplementation of vitamin C may improve the changes in the erythrocyte antioxidant system, which is damaged by OxS induced by exercise (Tauler et al., 2003). However, this does not work progressively ─ an excessive intake of antioxidant nutrients may have suppressive effects on immune reactions and other signalling functions (Niess et al., 1999).

In our study, serum vitamin C remained in normal baseline values in all study subjects (4-15 $mg \cdot l^{-1}$) (Inayama et al., 1996). An important finding of the present study was that changes in serum vitamin C after the competition were dependent on the relative heart rate during the competition (percentages of the maximal heart rate during the marathon). Thus, the subjects, whose physical effort was maximal, showed a higher increase of serum vitamin C. In addition, the present ski marathon was characterized by relatively difficult landscape and resulted in exhaustive strain (up to 87.8 % from maximal heart rate, Table 1).

According to the present data, an exercise-induced increase of glutathione redox status ($GSSG \cdot GSH^{-1}$) in whole blood and a decrease in $GSSG \cdot GSH^{-1}$ in erythrocytes clearly reveal that the antioxidant defence system may be effective in preventing the RBCs from the ROS (Margaritis et al., 2003; Ramel et al., 2004; Tauler et al., 2003).

In agreement with our findings, unchanged whole blood TGSH and GSSG levels and also reduced GSH and GSSG/TGSH ratio derived from those values by either acute exhaustive exercise or 8-week treadmill training in streptozotocin-induced diabetic rats were reported (Gul et al., 2003).

Conclusion

In conclusion, our data reveal that acute exercise, a ski marathon, slightly increases the redox status ($GSSG \cdot GSH^{-1}$) in whole blood, while it significantly decreases redox status in RBCs in endurance trained men. Our data show that exercise-induced changes in non-enzymatic components of the glutathione system (GSSG and GSH) in whole blood and RBCs are not identical. Thus, both analysis are needed to get more information about the glutathione system under exhaustive exercise.

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Key points

- The glutathione system is a principal cellular non-enzymic antioxidant system in the organism. Long-term or high-intensity exercise may lead to a decreased level of reduced glutathione (GSH), and thereby increase the glutathione redox ratio ($GSSG \cdot GSH^{-1}$).
- Limited data are available about the glutathione redox ($GSSG \cdot GSH^{-1}$) status measured simultaneously in red blood cells (RBCs) and blood concerning acute high-intensity exercise.
- Acute high-intensity exercise slightly increases the $GSSG \cdot GSH^{-1}$ in whole blood, while $GSSG \cdot GSH^{-1}$ significantly decreases in RBCs.
- Our descriptive data show that exercise-induced changes in the non-enzymatic glutathione system seem to be more effective in RBCs and may prevent the damages resulting from reactive oxygen species during exercise.

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