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# ANAEROBIC EXERCISE AFFECTS THE SALIVA ANTIOXIDANT/OXIDANT BALANCE IN HIGH-PERFORMANCE PENTATHLON ATHLETES

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ABSTRACT
**Purpose.** Investigate free radical production and antioxidant buffering in military pentathletes' saliva after their performance of a standardized, running-based anaerobic sprint test (RAST). **Methods.** Seven members of the Brazilian Navy pentathlon team were recruited to perform a running-based anaerobic test (~90 sec). The participants provided samples of saliva before and after the test that were analyzed for biomarkers of oxidative stress such as lipid peroxidation, total antioxidant capacity and the quantity of two specific antioxidants, glutathione and uric acid. **Results.** The lipid peroxidation increased ~2 fold after RAST, despite an increase in total antioxidant capacity (46%). The concentration of reduced glutathione did not change, while the uric acid concentration increased by 65%. **Conclusions.** The evaluation in saliva following a sprint test that lasted no more than 90 sec was sensitive enough to reveal changes in redox state.

**Key words:** saliva, physical exercise, oxidative stress, GSH, lipid peroxidation

## Introduction

Free radicals are not intrinsically harmful to health: low-to-moderate concentrations play multiple regulatory roles in gene expression, cell signaling, and skeletal muscle force production [1, 2]. However, if there is an imbalance between production of free radicals and antioxidant capacity, oxidative stress occurs and can provoke tissue damage [3, 4].

Anaerobic and aerobic exercise can both increase free radical formation. Prominent among mechanisms of free radical production during anaerobic exercise are mitochondrial leakage, ischemia-reperfusion response and leukocyte activation [5, 6], so a short burst of intense anaerobic exercise can be effective in generating oxidative stress as assessed by xanthine oxidase activity and markers for lipid peroxidation, protein carbonylation, and DNA oxidation, as well as total antioxidant capacity [5]. Strenuous aerobic exercise induces an increase in lipid peroxidation in human plasma [1], likely generated by hydroxyl radical attack on polyunsaturated fatty acids [5].

Providing blood samples increases stress in athletes that can limit their willingness to participate in scientific studies, especially during competition. This study examines oxidative stress, for the first time in military athletes training for an international competition called the naval pentathlon, using a non-invasive analysis of saliva. The naval pentathlon consists of five different tests performed on consecutive days. The tasks require intense anaerobic effort and in some cases apnea. There is very little published about reactive oxygen species (ROS)/antioxidant balance in saliva of high-level anaerobic athletes. We hypothesized that saliva samples are suitable as biological material to monitor changes in biomarkers for free radical generation and total antioxidant capacity. Saliva samples were collected before and after a short test, RAST (running-based anaerobic sprint test), and used to assess anaerobic performance during the training period [7].

## Material and methods

### Subjects

Subjects were seven military athletes of the Brazilian Navy, all of them experienced in international competition in naval pentathlon. They were engaged in the final weeks of training for a series of international tournaments. The team with five men and two women volunteered to participate in the study. Their written informed consents were obtained after explanation of the purpose, benefits and potential risks to the subjects. All military personnel are submitted to periodic medical and odontological examinations. The results of the oral health evaluation were used to exclude any athletes

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showing symptoms of oral inflammation, periodontitis or gingivitis.

The diet of all participants during the week prior to testing was the same and obtained from the military facility, which was controlled by a military nutricionist to meet the energy demands of the armed services. Subjects were instructed not to ingest supplemental vitamins or antioxidants. In addition, each was instructed to avoid smoke and heavy physical exercise for 1h prior to testing. The experimental protocol was approved by the Ethics Committee of Clementino Fraga Filho Hospital of the Federal University of Rio de Janeiro.

### Aerobic capacity and anthropometric measurements

Maximal oxygen consumption ($VO_{2max}$) was estimated on a synthetic outdoor track several days before the RAST, applying the distance run in 12 min to the Cooper [8] regression equation. The results are presented in relative rate in milliliters of oxygen per kilogram of body weight per minute (Table 1). Skinfold thickness was measured as described in Pollock and Jackson [9]. Body fat percentage was determined using the Siri [10] equation.

### Exercise test

The RAST, a single set applied individually, was performed outdoors on grass. It consisted of 5 min of very light warm-up (stretching and jogging) followed by 6 × 35-m sprints as fast as possible, with 10 s between sprints, and the last sprint followed by 5 min of cool-down. The power generated in each sprint was calculated by the formula Power (W) = (Body mass × Distance$^2$)/Time$^3$, normalized to body weight (Table 2).

Table 1. Anthropometric characteristics and aerobic capacity

| | Mean ± *SD* | Range |
|---|---|---|
| Age (yr) | 27.1 ± 5.4 | 21–31 |
| Body mass (kg) | 65.3 ± 6.6 | 53.8–72.2 |
| Height (cm) | 170 ± 10 | 160–185 |
| BMI* (kg/m$^2$) | 21.9 ± 0.8 | 20.7–23.2 |
| Body fat (%) | 10.2 ± 5.6 | 3.9–20.3 |
| $VO_{2max}$ (mL/kg · min) | 61.9 ± 12.0 | 44.6–73.7 |

* body mass index = mass(kg)/(height(m))$^2$

Table 2. Measures of performance in the RAST

| | Mean ± *SD* | Range |
|---|---|---|
| Peak power per weight (W/kg) | 6.5 ± 1.4 | 4.4–7.9 |
| Average power per weight (W/kg) | 5.4 ± 1.1 | 3.8–6.7 |
| Minimum power per weight (W/kg) | 4.6 ± 1.1 | 3.0–5.9 |

Power (in watts) is normalized to body weight (kg).

### Collection of saliva

To avoid contamination, the subjects washed their mouths with deionized water before the collection and then chewed a piece of cotton wool for 1 min. Saliva samples were collected before and 5 min after the RAST as suggested by several papers [11–13]. After being collected, the samples were transported on ice to the laboratory and centrifuged at 3,000 × *g* for 10 min at 4°C. Supernatants were separated from pellets and stored at – 20°C.

### Lipid peroxidation assay

The lipid peroxidation assay was performed as described by Zalavras et al. [14] with slight modification. One hundred microliters of saliva supernatant was mixed with 500 µL TCA (35%, w/v) and 500 µL Tris-HCl (200 mM, pH 7.4) and incubated for 10 min at room temperature. One milliliter of a solution containing 55 mM thiobarbituric acid in 2 M $Na_2SO_4$ was added and the samples were incubated at 95°C for 45 min and then cooled on ice for 5 min. After the addition of 1 mL TCA (70%, w/v) they were vortexed and centrifuged at 15,000 × *g* for 3 min. The absorbance of the supernatant was read at 530 nm and TBARS concentration was calculated using an extinction coefficient of e = 0.156 $\mu M^{-1} \cdot cm^{-1}$. Values were expressed in µM.

### Total antioxidant capacity

The total antioxidant capacity was measured as in Georgakouli et al. [15], using 20 µL saliva, 480 µL sodium-potassium phosphate (10 mM, pH 7.4) and 500 µL 2,2-diphenyl-1-picrylhydrazyl (DPPH, 0.1 mM), incubated in the dark for 30 min and centrifuged for 3 min at 20,000 × *g*. The absorbance $A_c$ was read at 520 nm and compared with $A_0$, absorbance of a reference sample containing only 20 µL water, DPPH and buffer. Percentage reduction of the DPPH ($Q$) was defined by $Q = 100(A_0 - A_c)/A_0$ [16].

#### *Determination of GSH in saliva*

One hundred microliters of saliva was added to 200 µL of a 10% solution of TCA, vortexed and centrifuged at 4,000 × *g* for 10 min at 10°C. To 200 µL of the supernatant was added 700 µL of 400 mM Tris-HCl buffer, pH 8.9, followed by 100 µL of 2.5 mM DTNB dissolved in 40 mM Tris-HCl buffer, pH 8.9. The samples were incubated for 10 min at room temperature and the absorbance was measured at 412 nm. Blanks contained water instead of saliva. The concentration of GSH in the samples was read from a GSH standard curve (0.8 µM – 4 µM) [17].

Uric acid in saliva

Urate concentration in saliva was analyzed by a uric acid assay kit (Doles Urato 160, Goiania, GO, Brazil) based on the amount of $H_2O_2$ produced when urate is converted to allantoin by uricase.

Statistical analysis

Pre- and post-test samples were compared using Student's paired *t*-test. Data normality was verified with the Shapiro-Wilk test, which showed that non-parametric test was not required. The power of the performed tests with an $\alpha = 0.05$ was 1.000 to TBARS, 0.735 to DPPH, 0.999 to uric acid and 0.098 to GSH. Correlation between variables were assessed by Pearson's correlation coefficient. For all analyses, statistical significance was indicated when $p < 0.05$. Standard errors are reported, except for anthropometric characteristics, aerobic capacity and measures of performance in the RAST.

**Results**

Correlation between aerobic and anaerobic performance

The subjects showed a high $VO_{2max}$, as presented in Table 1. Anaerobic power was assessed during each RAST sprint (Table 2). The correlation coefficient between peak anaerobic power and previously determined $VO_{2max}$ for each subject was high ($r = 0.94$, Figure 1).

Lipid peroxidation in saliva

In this study the peroxidation of fatty acids was assessed by an assay for thiobarbituric acid-reactive substances (TBARS). After the RAST, the lipid peroxidation was ~2 times higher than at rest (Figure 2). In the pre-test condition the value obtained was 0.9 µM ± 0.2 µM and five minutes after six sprints the value was 1.9 µM ± 0.2 µM.

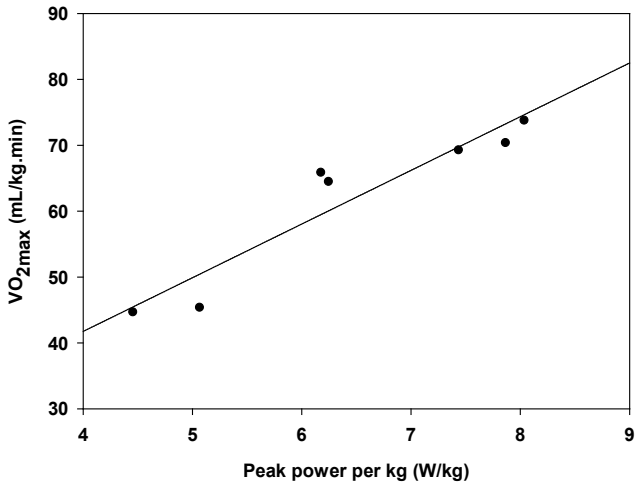


Figure 1. Correlation between aerobic and anaerobic power for each athlete presented a R2 = 0.8885 and a correlation coefficient of 0.94 ($n = 7$), $p < 0.05$

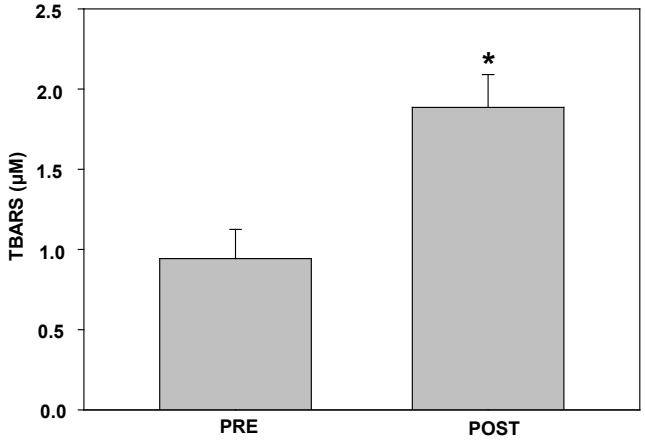


Figure 2. The anaerobic test RAST induces oxidative stress. Mean ± S.E. ($n = 7$), *$p < 0.05$

Total antioxidant capacity

To evaluate the overall antioxidant response to the RAST, which as shown above generated a substantial lipid oxidation, we measured the quenching of DPPH absorbance after the addition of saliva. This measure of total antioxidant capacity increased by 46.6% (Figure 3).

Non-enzymatic antioxidant system

To evaluate the contribution of the non-enzymatic antioxidant system to the increment observed in total antioxidant capacity, we evaluated the salivary glutathione and uric acid. There was no significant change in glutathione status (Figure 4A), but uric acid increased by 65.6%, from 178.9 µM ± 21.4 µM to 293.5 µM ± 9.4 µM (Figure 4B).

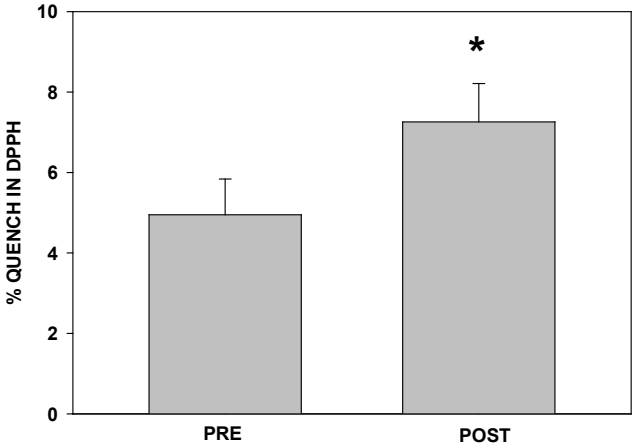


Figure 3. RAST increases the total antioxidant capacity in athletes' saliva. Mean ± S.E. ($n = 7$), *$p < 0.05$

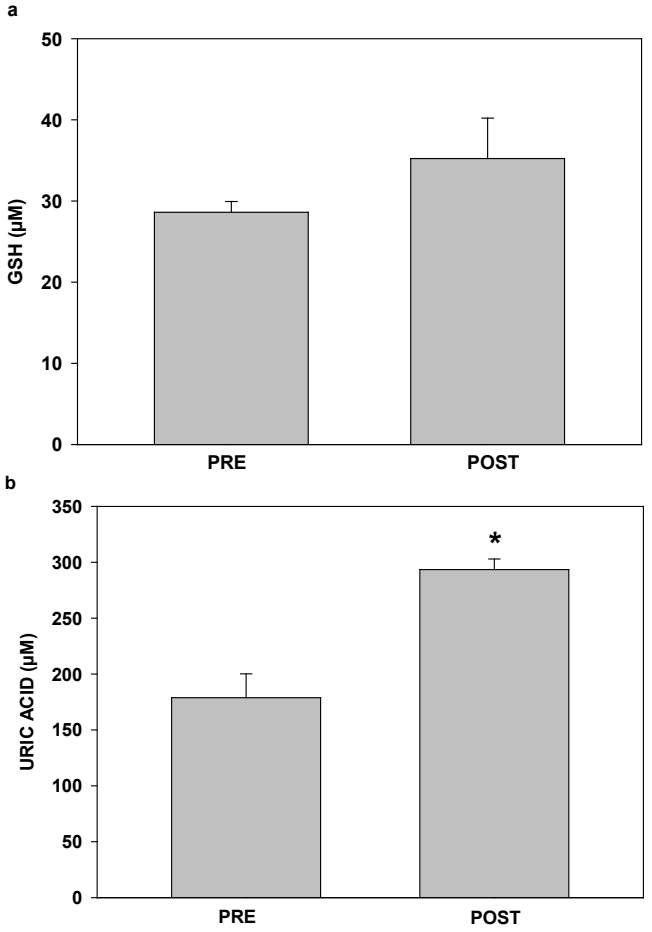


Figure 4. Antioxidant biomarkers. A) Glutathione (GSH); B) Uric acid. Mean ± S.E. ($n$ = 7), *$p < 0.05$

## Discussion

The aim of this study was to investigate free radical production and antioxidant buffering in military pentathletes' saliva after an anaerobic test, RAST. The running-based anaerobic sprint test is designed to assess anaerobic power in sports that mostly use running [7].

The athletes evaluated had a high $VO_{2max}$, commonly accepted as a key indicator of the endurance capacity. The two women averaged 45.0 ± 0.5 mL/kg · min and the men 69.0 ± 4.0 mL/kg · min. The values for the men were equivalent to those reported for male triathletes (67.6 ± 4.5 mL/kg · min) [18] and a European team of naval pentathlon athletes (74.0 mL/kg · min) [19].

The naval pentathlon is an intense sequence of five anaerobic tasks performed on consecutive days: obstacle race, life-saving swimming race, utility swimming race, seamanship race and amphibious cross-country race. They range from about 60 s (life-saving race) to 10–12 min (cross-country race). The RAST is an intense anaerobic test but it is short and the effort level is lower than the actual naval pentathlon competition. Other authors have studied metabolic parameters after application of a modified RAST protocol, but they did not measure redox status [20]. The RAST has not been exploited for assessment of biochemical changes in athletes, although it is frequently used to predict running performance, with high correlations for 35, 50, 100, 200 and 400 meters [7]. During a pentathlon competition the distance covered by running varies in different tests from 280 to 900 meters. Although the RAST is shorter it was able to provide a measure of pro-oxidant and antioxidant status in well-trained athletes in a controlled test condition.

In an intermittent high-intensity test such as the RAST, major sources for ATP replacement are initially phosphocreatine hydrolysis and glycolysis, but a shift toward oxidative metabolism to replenish ATP has been demonstrated for later trials in a sequence of sprints [21]. We observed a high correlation between $VO_{2max}$ and anaerobic power, consistent with a role for aerobic power in anaerobic performance, and suggesting the importance of such a shift during the RAST, as observed for other anaerobically trained athletes, for example, judo players performing an anaerobic judo test that lasted 60 s [22].

Very few studies have proposed saliva as an alternative source to evaluate oxidative stress biomarkers and antioxidant adaptation induced by exercise [11], and Deminice et al. [12] emphasized the differences between redox profiles of plasma and saliva. However, the main finding in our study was that saliva of trained athletes, following an anaerobic test, reveals an increase in lipid peroxidation (TBARS) and at the same time an increase in the antioxidant capacity that can be attributed primarily to uric acid (UA) – consistent with published data obtained using serum or plasma. Under hydroxyl radical attack the double bonds in polyunsatured fatty acid of biological membranes can degrade membrane structure with loss in physiological function and cellular disruption [2, 23]. The increase in lipid peroxidation after six sprints shows that mechanism for free radical production can override antioxidant defenses even in highly trained athletes, and this can be seen clearly in saliva.

After the RAST, the quenching of DPPH, a measure of total antioxidant capacity, was 46% higher than at rest (Figure 3). These data show that although these athletes undergo substantial lipid peroxidation (Figure 2), the total antioxidant capacity was not used up.

Wayner et al. [24] have assigned total peroxyl trapping in human plasma to uric acid (35–65%) and the thiols of plasma proteins (10–50%), with minor roles for ascorbic acid and vitamins. An increase in TBARS and/or UA concentrations post-exercise has been found in other studies [11, 12, 25], but in agreement with our observations there were no large changes in GSH. It is worth noting that a large decrease in GSH was recorded in the cases where whole blood was analyzed [25], suggesting that the use of saliva has the advantage of avoiding artifacts due to hemolysis or contamination with erythrocytes.

In our study the GSH concentration did not change (Figure 4A), as also reported by Deminice et al. [12], who tested saliva of healthy well-trained males after a 40-min session of anaerobic resistance exercise. Even prolonged aerobic exercise of moderate intensity was not able to alter GSH concentration in skeletal muscle in healthy adults [26]. Thus GSH appears to be tightly regulated, with secretion from liver to plasma designed to ensure homeostasis in blood [27]; in our study the concentration in saliva also appeared to be tightly controlled. In another study that assessed anaerobic resistance training, Margonis et al. [25] found a decrease in GSH concentration in the blood only in association with overtraining. Wiecek et al. [28] did not measure any alteration in GSH plasma concentration 3 min after the anerobic exercise, which is in line with the saliva meaurements presented here, although the authors showed a reduction after 15 min up to 24h. Since our data only includes a collection at 5 min post aerobic exercise, this time dependent effect cannot be ruled out. Further experiments need to be performed to evaluate the time dependent behavior of GSH levels in saliva.

Physical exercise triggers antioxidant adaptations [1, 29] that upregulate expression of endogenous antioxidant enzymes such as eNOS, MnSOD and iNOS expression [1, 30] as well as increases the non-enzymatic antioxidants [2]. Foti and Amorati [31] showed that uric acid accounted for one-third of the increase in antioxidant capacity suggesting that our observed increment in uric acid content in saliva represented an increase in total antioxidant capacity. Electron spin resonance and chemical studies indicate that uric acid can react with peroxyl radical, providing scavenging abilities [31]. This chemical feature may mean that uric acid helps to control oxidative stress generated by exercise, at the same time reflecting greater ATP flux associated with elevated energy requirements. Purine catabolism increases and the higher concentrations of sub-products of this metabolism are formed [32]. Xanthine oxidoreductase is a key intracellular enzyme of purine metabolism. Its oxidase form is responsible for converting hypoxanthine to xanthine and xanthine to acid uric [33]. The purine cycle generates superoxide, hydrogen peroxide, and the reactive hydroxyl molecule [34], but uric acid itself is a potent non-enzymatic antioxidant [25]. Formed in the liver from hypoxanthine, it is released to the blood and either excreted or taken up by muscle, where it scavenges hydroxyl radical [35].

## Conclusions

Based on the results of this study, it can be concluded that this study shows that it is possible to obtain a profile of redox state using a non-invasive approach associated with a short anaerobic test. RAST triggers free radical production, as evaluated by lipid peroxidation in saliva, and at the same time reveals an increasead antioxidant capacity as a sub-acute adaptation to a short series of sprints.

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