

Salivary antioxidant variations in athletes after intense exercise

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Abstract. *Introduction.* Antioxidant system in saliva protects oral cavity as well as gastrointestinal tract from attack of free radicals. The aim of this research was investigating the whole antioxidant system variations in saliva of athlete men. The effect of short term intense exercise on salivary flow rate, superoxide dismutase (SOD) activity and uric acid concentration was examined. *Material and Method.* In a double blind randomized design, 25 healthy athlete men (mean age 22.9±1.5 years, mean weight 73.77±5.8, height 176.6±7.13m body fat 14.34±1.31% and maximum oxygen consumption 48) enrolled for the research. Before collecting their un-stimulated saliva, the purpose of study was explained and informed consent obtained. Their un-stimulated whole saliva samples were collected before, immediately after and one hour after treadmill runs at exhaustion. Activity of antioxidant enzymes (catalase, peroxidase, superoxide dismutase) and concentration of uric acid were determined using corresponding biochemical assays. *Results.* The results showed a significant increase in uric acid concentration and activity of enzymes immediately after exercise. However, this increase was slowly compensated in one hour after exhaustion. *Conclusions.* Therefore, short-duration and high-intensity exercise increases the antioxidant power of saliva for scavenging the free radicals produced by stress from intense exercise.

Key words: athletes, saliva, flow rate, salivary whole antioxidants.

Introduction

It is well established that various exercises could reduce the risk of many diseases including diabetes, cancer and cardiovascular disorders. However, there are some evidences on increasing the reactive oxygen (and/or nitrogen) species during intense exercise (1, 2). During an intense exercise, the body's oxygen demand may increase to about 15-20 times the normal value, due to the increase in mitochondrial oxygen consumption (3). Reactive oxygen species (ROS) could cause oxidative stress and increase the rate of oxidation. Natural antioxidants present in various body sites, could reduce the oxidative damage by scavenging the free radicals (4, 5). There are also exogenous antioxidants which are provided through food consumption (6, 7) The later class of antioxidants is usually non-enzymatic chemical compounds, such as ascorbic acid. Moderate exercise could reduce risk of various infections due to increased body resistance (8). A body of research has indicated that various physical conditions are associated with increased resistance to infection (9). However, it has been stated that athletes of chronic high intensity training are in high risk of

upper respiratory tract infection (10). It is suggested that rise of infection in athletes could be related to a decrease in salivary immunoglobulin-A (11).

Saliva is the first body fluid to encounter different solids, fluids and gases entering the gastrointestinal tract. Human saliva is composed of various natural defense mechanisms including the immunological and antioxidant defense systems (12). A significant increase in secretion rates and absolute concentrations of salivary antimicrobial proteins and markers has been reported during intense training and competition period as well as the first week of recovery (1). Salivary immunoglobulin has shown variations in children infected with *Streptococcus mutans* (13) and in swim trainers (11). Up to now, many research works have used blood plasma to study body's response to exercise. However, the use of saliva as a non invasive research fluid is increasing more recently (12, 14). On the other hand, many important proteins and various ions could be transferred from blood to saliva by special mechanisms of salivary glands (15).

In addition, measurement of serum biochemicals requires venepuncture, which is associated with negative feelings and oxidative stress, leading to altered values (4, 16). Therefore, human saliva containing many important compounds could be a reliable representative of both salivary gland function and plasma composition. Salivary antioxidants are composed of catalase, peroxidase, superoxide dismutase, uric acid, and low amounts of fat soluble vitamins, A and E which are contained in lipoprotein systems (17, 18). The importance of salivary antioxidant enzymes is due to their power for decomposing various free radicals entering the oral cavity as well as the H_2O_2 produced by oral bacteria. The most important antioxidant enzyme of saliva, peroxidase, alters significantly due to intense exercise (19). In a previous study alternations in enzymatic and non-enzymatic antioxidant systems of saliva were reported for non-athletes (20). The aim of this research was to further examine changes of enzymatic and non-enzymatic antioxidants in saliva of healthy athletes. Therefore, alternations in oral peroxidase, superoxide dismutase and uric acid in salivary fluid of athlete men after a short period intense exercise were examined. The hypothesis was that there must be changes in antioxidant capacity of saliva due to short period intense exercise.

Material and Method

The necessary chemical reagents were of analytical grade and used as provided by manufacturers, no purification was needed. Superoxide dismutase kit was purchased from Cayman chemical, Cat No.706002, USA. Activity of peroxidase on 4-amino antipyrine and H_2O_2 was measured spectrophotometrically.

4-Amino antipyrine, phenol, hydrogen peroxide, horseradish peroxidase were purchased from Merck chemical company. Salivary uric acid kit was purchased from local representatives in Iran. The necessary solutions and buffers were prepared freshly using double distilled water in Research Laboratory of Biochemistry, University of Guilan. All chemicals, solvents and reagents were obtained with highest purity available.

The subjects of the study were 28 healthy male athlete university students (mean \pm SD; age = 22.9 ± 1.5 years; height = 176.6 ± 7.13 cm; mass = 73.77 ± 5.8 kg; VO_2 peak = 48.3 ± 3.63 mL kg^{-1} min^{-1} ; maximal heart rate = 191.4 ± 3.7 beats min^{-1} ; body fat = $14.34 \pm 1.31\%$). They received local ethics community (Helsinki) approval before

agreeing to enter the study (Table I). Volunteers were informed about the aims of the study before providing written informed consent and were asked to fill in a comprehensive health questionnaire.

Table I. Main characteristic of subjects run for 20 minutes at heart rate values which corresponded to 80% VO_{2max} .

Age (yrs)	Mass (kg)	VO_{2max} (ml/kg ¹ /min ⁻¹)	Height (cm)	Body fat (%)
Mean: 22.9	73.77	48.3	176.6	16.36
SD: 1.5	5.8	3.63	7.13	1.31
Range: 20-27	59.5-75.3	41.3-52.6	169-183	10.1-20.3

The study was based on a randomized, double-blind, cross-over design. All subjects performed three trials; before, immediately and one hour after experimental trials. The first saliva sample was collected 5 minutes before treadmill run and the next two 5 minutes and one hour after trial respectively. Before donating their un-stimulated saliva samples, the volunteers had brushed their teeth and rinsed their mouth with distilled water. When the first saliva sample was collected in marked tubes, the subjects performed treadmill run using Astrand test at 8.01 km/h followed by the next two collections 5 minutes and one hour after the trail.

Measurement of VO_{2max} . All subjects performed a continuous incremental treadmill run to exhaustion. The test began at a velocity of 8.05 km/h, with an increase of 3.0% km/h every 3 minutes until exhaustion. Gas change parameters were analyzed during the run by a calibrated Sormedics Horizon Metabolic Measurement Cart (Sormedics, Anaheim, Calif). All through the whole trail, their heart rate was recorded every minute.

Saliva collection, storage and determination of flow rate. Timed un-stimulated whole saliva samples (3 ml) were collected in clean, dry in sterile pre-weighted tubes. The duration of saliva sampling was altered among individuals depending on their flow rate (2.0-5.0 minutes). The flow rate was calculated by measuring the time (in minutes) taken for collection of one ml saliva samples. The saliva samples were immediately centrifuged at $900 \times g$ for 10 min at $4^\circ C$ to remove squamous cells and cell debris.

The resulting supernatant was stored at -20°C until assayed for antioxidants activity.

Determination of salivary uric acid. The amount of uric acid was measured by an enzymatic method described for assay of uric acid in serum (21). The complete assay was based on enzymatic reaction of uricase on uric acid to form allantoin and hydrogen peroxide (H_2O_2). Production of hydrogen peroxide was coupled with catalytic oxidation of p-hydroxybenzoate and 4-aminoantipyrine oxidation in the presence of peroxidase. The pink chromophore thus formed was then detected at 505 nm.

Measurement of catalase activity. One unit of catalase activity is defined as the amount of enzyme that decomposes one micromole of hydrogen peroxide in minute at pH 7.0 (22). The enzyme activity was measured using hydrogen peroxide in phosphate buffer (pH 7.0). The absorption of mixture was monitored at 240 nm at 10 second intervals during 2 minutes. The obtained absorbances were then divided by 39.4 to obtain catalase activity.

Salivary peroxidase assay. The biological activity of peroxidase on 4-amino antipyrine was measured spectrophotometrically. The oxidation of 4-amino antipyrine was measured at 25°C in 3 ml of 0.3 M phosphate buffer, pH 7.4, containing 0.0010 M hydrogen peroxide, 0.002 M 4-amino antipyrine and 0.15 M phenol. 40 μl of enzyme solution (6×10^{-4} mg/ml in 0.3 M phosphate buffer pH 7.4) was then added and the change in absorption at 510 nm was recorded. The change in absorption at 510 nm is due to the formation of a chromogen product. The statistical differences are given in the result section. One unit of activity is the amount of enzyme that caused an absorbance change of 0.001 per min under standard conditions.

Measurement of superoxide dismutase activity. Salivary superoxide dismutase activity was measured by an enzyme assay kit (Cayman chemical, Cat No.706002, USA). According to procedure given in the kit, tetrazolium salt was used for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was the amount of enzyme needed to exhibit 50% dismutation of superoxide radicals. The whole experimental section was performed by an experienced technician blind about cases.

Statistical analysis. All enzymatic and non-enzymatic antioxidant measurements were

repeated at least in triplicate and the results were presented as mean \pm SD.

Statistical difference between individuals was compared by un-paired t-test. Significant differences between means were determined by Duncan's multiple range tests. *P* values less than 0.05 were considered statistically significant.

Results and discussions

Salivary flow rate. Table II has compared the results of flow rates before and after exercise. As stated in the experimental section, the salivary flow is volume of un-stimulated saliva (ml) collected per minute. Although slight alternations were observed between saliva flow rate before and after exercise, the rate was not significantly altered. It was found that the saliva flow rate ranged from 0.75-0.42 $\text{ml}\cdot\text{min}^{-1}$ ($n=28$) before exercise, and not significantly changed immediately ($n=28$, 0.73-0.44 $\text{ml}\cdot\text{min}^{-1}$) after intense exercise. This is an interesting result as it indicates that dehydration during exercise does not seriously affect the normal flow rate of saliva. It has been reported that the flow rate of other biological fluids such as tears (23, 24), may change due to various external and internal factors. Salivary fluid has also shown variations in flow rate (5). However, the athletes could better resist fluctuations in external factors (1). In contrast to the present results, we have previously found a significant decrease in salivary volume and rate of flow in smokers that is due to the presence of various toxic chemicals in cigarette smoke (25). This result can explain why heavy smokers complain from dry mouth even at young ages. Salivary volume and flow rate is of prime importance in maintaining a healthy environment for oral cavity and preventing dry mouth syndrome. It can be concluded that a regular exercise of almost any type could also be beneficial to oral health.

Table II. The flow rate of saliva in athlete men after a short intense exercise

Before exercise (n=28)	Immediately after exercise (n=28)	One hour after exercise (n=28)
0.48 \pm 0.07	0.47 \pm 0.12	0.49 \pm 0.13
0.35-0.74	0.29-0.85	0.33-0.76

In agreement to the present research, it has been reported that salivary flow rate does not significantly alter in response to short period and medium or high intensity exercise (1, 19).

However, some significant decrease in flow rate has also been reported (26, 27). Disagreement between results could be related to lower activity of para-sympatic nervous system in short period low intensity exercise (27).

On the other hand, dehydration up to about 2% of whole body weight in high intensity exercises could also lead to lower flow rate of saliva (28). Therefore, in the case of athletes, dehydration or stimulation of nervous system has not caused severe reduction in their saliva flow rate.

We have found that the flow rate in non-athlete men is decreased after intense exercise (20). The decrease of flow rate in non athletes is due to the high intensity exercise trail which has not been experienced in non-athlete men (1).

Many other factors including age (29, 30) and gender (31) may influence the flow rate of un-stimulated saliva. For example, the flow rate in women is lower than men due to the smaller sizes of their salivary gland compared to men (31).

It has also been reported that the unstimulated and stimulated flow rates of submandibular saliva change during the menstrual cycle and are highest during the follicular phase (32).

However, in the present study, no significant difference in the un-stimulated salivary flow rates of athletes was observed.

This finding indicates that as many other body conditions, the oral fluid of athletes could also resist severe alternations.

Enzymatic and non-enzymatic antioxidants.

Figure 1 shows variations in uric acid concentration due to exercise. Figures 2-4 show alternations of three enzymes, catalase, peroxidase and superoxide dismutase in saliva of athletes respectively. The only non-enzymatic antioxidant measured in this study was water soluble, uric acid. As seen in these figures, both enzymatic and non-enzymatic antioxidants increased immediately almost significantly after exercise. However, their activity was returned to their normal value one hour after exercise. Uric acid is the most important non-enzymatic antioxidant in human saliva. It is entirely water soluble and induces its antioxidant action much more quickly than fat soluble antioxidants (33, 34). In contrast to present finding for athletes, concentration of salivary uric acid showed a decrease immediately after intense exercise in non-athletes (20). However, in that case flow of saliva after a short period has returned the uric acid concentration to its base line level. In athletes, on the other hand, which have higher flow rates (0.48 ± 0.07 compared to 0.41 ± 0.09), uric acid was increased and returned to normal after an hour. Activity of all three enzymes was higher after immediately exercise, as stress could induce the enzymatic activity. This result is in agreement with our previous findings on alternations in activity of salivary peroxidase in response to exercise intensity (10) and cigarette smoking (25).

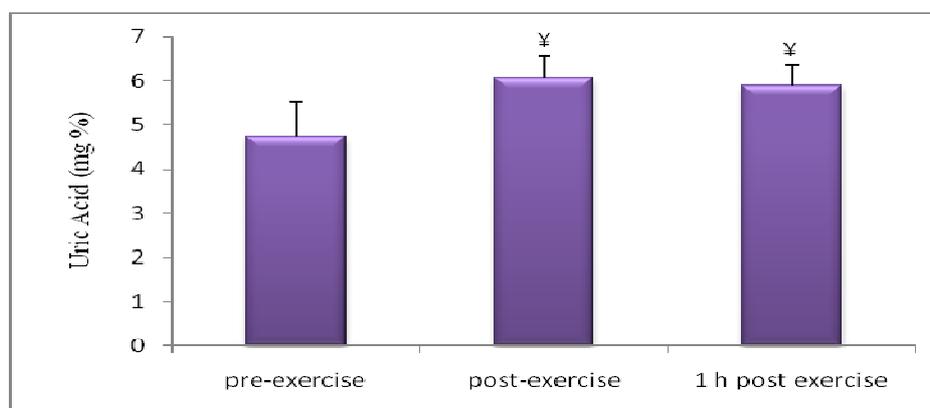


Figure 1. Variations of salivary uric acid concentration due to treadmill run in athletes.

¥: Significantly different from pre-exercise $p \leq 0.05$.

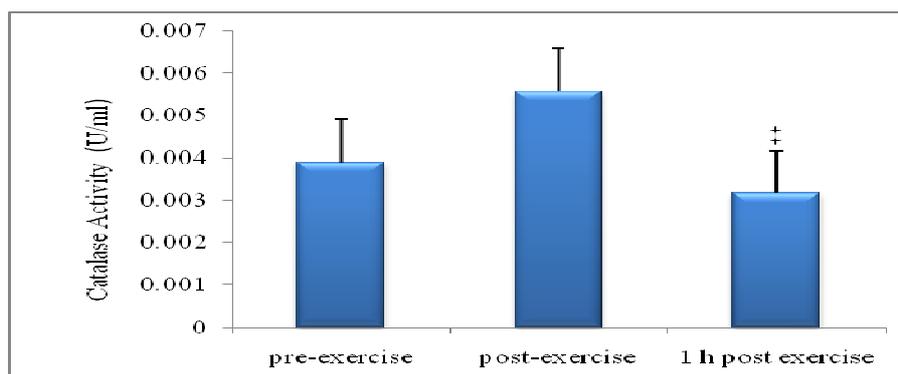


Figure 2. Variations of salivary catalase due to treadmill run in athletes.

‡: Significantly different from post-exercise $p \leq 0.05$.

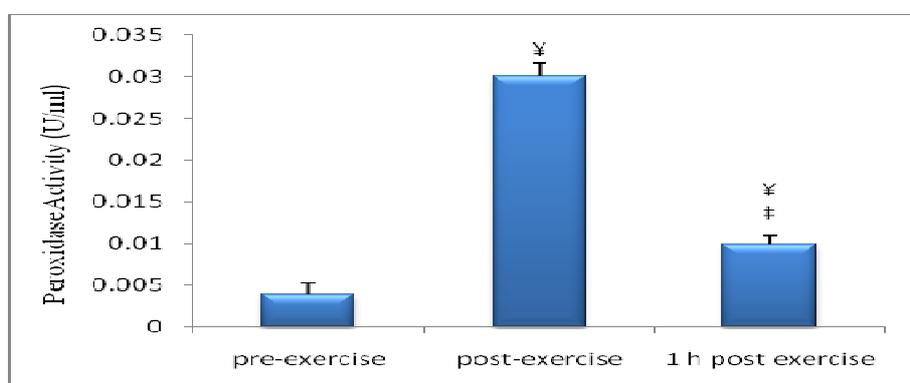


Figure 3. Variations of salivary peroxidase due to treadmill run in athletes

‡: Significantly different from pre-exercise $p \leq 0.05$.

‡: Significantly different from post-exercise $p \leq 0.05$.

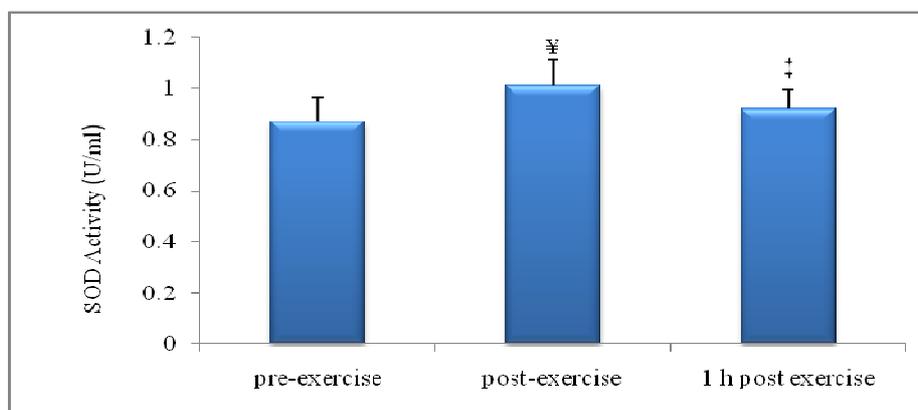


Figure 4. Variations of salivary superoxide dismutase due to treadmill run in athletes

‡: Significantly different from pre-exercise $p \leq 0.05$.

‡: Significantly different from post-exercise $p \leq 0.05$.

The salivary antioxidant system is composed of various molecules and enzymes. The most important antioxidants in human saliva are water soluble uric acid, peroxidase, superoxide dismutase and ascorbic acid respectively (35). The concentration of lipid-soluble antioxidants is very low, contributing only 10% of the total salivary antioxidant capacity (36, 37).

It has been reported that the uric acid is responsible for about 70% of the total salivary antioxidant capacity (36). The present study showed that salivary uric acid (Figure 1) was significantly increased immediately after exercise, 6.07 compared to 4.73 mg%. In contrast to these values, uric acid in stimulated saliva has been reported to be lower than this value (38).

This finding proves that stimulation of saliva can affect the antioxidant capacity. The value obtained here are close to the uric acid concentration in unstimulated saliva (39). Uric acid acts as a chelating agent and prevents the attack of free radicals as well as being a scavenger of the generated free radicals. Figures 2-4 indicate that activity of catalase, peroxidase and SOD is increased almost significant immediately after intense exercise which returned near to original value one hour after exercise. The return of these important enzymatic antioxidants to normal activity is vital for providing total protection for oral cavity in general. Many researchers have indicated the beneficial effects of aerobic exercise on human general health (40). On the other hand, it has been found that long period exhausting activities increases cellular ability to prevent the attack of free radicals [4]. However, some evidences have shown that the antioxidant activity of human body does not significantly alter even after long period high intensity exercise (41). This discrepancy could be related to the individual responses to external stimuli and further research is still needed. It is known that aerobic exercises lead to increased production of reactive oxygen species (ROS) such as superoxide, and hydroxyl radicals. Therefore, higher production of antioxidants during this type of activity is a type of biological response for deactivation. Increase in enzymatic activity has also been reported in saliva of elite judoist after exercise (14). That study has only examined activity of SOD and has expressed that the type of stress may cause variations in antioxidant status of saliva. The increase in enzymatic activity has been reported in saliva samples in children playing computer games. It has been found that even after 3 hours the antioxidant enzyme activity has not returned to the normal value before game. This result is surprising and entirely different from our study and could prove the high oxidative stress induce by many computer games (42).

Conclusions

Based on the results obtained in this study, it was found that aerobic exercise until exhaustion increases the activity of superoxide dismutase in saliva of athlete men. However, the most important water soluble salivary antioxidant, uric acid is not significantly affected by aerobic trials. It can be concluded that exercise induces production of free radicals and various reactive oxygen species. Increase of ROS in saliva causes

an increase in the activity of antioxidant enzymes including catalase, peroxidase and superoxide dismutase. The increase in concentration of uric acid is not significant due to its high concentration in human saliva. The activity of salivary antioxidant enzymes including catalase, peroxidase and superoxide dismutase is of prime importance in protecting oral cavity. Excess production of reactive oxygen species may lead to dangerous disorders not only in mouth itself, but also in the whole gastrointestinal tract. It could, in conclusion, be predicted that exercise at a moderate intensity could increase antioxidant capacity of saliva as well as other natural body fluids.

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