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Original Article

Salivary antioxidants of male athletes after aerobic exercise and garlic supplementation on: A randomized, double blind, placebo-controlled study

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ABSTRACT

Purpose: Production of reactive oxygen species and reactive nitrogen species is a natural biological event in metabolism. However, the presence of antioxidants can highly reduce the negative effect of free radicals. Thus, the efficiency of antioxidant system in the physiology of exercise is very important.

Design: Considering the known antioxidant capacity of garlic, the purpose of this study was to evaluate the effect on combining 14 days aerobic exercise till exhaustion with garlic extract supplementation on the antioxidant capacity of saliva.

Methods: Sixteen young men volunteered to participate in this randomized, double blind, placebo-controlled study and were randomly placed into two groups, placebo (Group I) and garlic extract (Group II). The participants performed exhaustive aerobic exercise on a treadmill before and after supplementation. Their unstimulated salivary samples were collected before, immediately after, and 1 h after the activity. The antioxidant activity in terms of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) was then measured in the collected samples using their specific substrates.

Results: A significant increase in salivary antioxidant activity of SOD, POD, and CAT was observed in saliva of the supplement group compared to the placebo group ($P \leq 0.05$).

Conclusion: The findings from this study suggest that increased activity of antioxidant enzymes could possibly decrease exercise-induced oxidative damage in male athletes.

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Abbreviations: CAT, catalase; EDTA, ethylene diamine tetraacetic acid; GTE, green tea extract; NBT, nitro blue tetrazolium; NF, nuclear factor; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity; TNF, tumor necrosis factor; TT, tapering training.

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1. Introduction

Many research studies have revealed that moderate and regular exercise has a number of benefits on the individual's health including reduced prevalence of cancer and cardiovascular disease.[1] However, there are minor evidences showing that severe activities may, sometimes, induce apoptosis in healthy cells, cancer, aging, damages to lipids, proteins, and DNA,[2] attenuation, and depletion in body antioxidant capacity through production of free radicals and reactive oxygen species (ROS).[3,4] Physical activity can cause an imbalance between ROS production and antioxidants, named oxidative stress.[5] Antioxidants can neutralize negative effects of free radicals and ROS, and also, decrease effects of oxidative stress.[6] Endogenous enzymatic antioxidants include glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD) and exogenous antioxidants include uric acid, coenzyme Q10, bilirubin, albumin, and vitamin A, C, and E.[7]

Inadequate antioxidant defense system may increase oxidative stress, which is accompanied by metabolic changes, and leads to poor performance in athletes.[8] Three proposed mechanisms of free radicals production associated with athlete are (1) increase in the rate of aerobic metabolic processes, (2) ischemic reperfusion, and (3) tiny injury to muscle and injury restoration.[9] It has been shown that neutralized ROS by antioxidant supplements can have a positive effect on exercise performance. On the other hand, use of edible and natural antioxidant supplements is one of the methods to counter against undesirable effects of oxidative stress induced by free radicals.[10] In this regard, some of the professional and amateur athletes believe that they can improve their performance through the use of antioxidant dietary supplements.[11]

SOD, the main enzyme to scavenge superoxide radicals, is the first line of defense against oxidative stress. SOD family of enzymes has a role in the superoxide catalysis on hydrogen peroxide production. Peroxidase (POD), present in the cytosol of cell and mitochondria, has the ability to reduce the damage of hydrogen peroxide by converting H_2O_2 to H_2O. On the other hand, CAT, which is present in the cells especially peroxisomes, could decrease fatigue-induced ROS through conversion of H_2O_2 to H_2O and oxygen.[7,12]

Saliva is a suitable body fluid for measurement of many enzymes, hormones, and other substances due to its easier availability, non-invasive nature, lower risk of blood diseases and infections, and the possibility of safely freezing and storage of samples.[13,14] On the other hand, as saliva is very similar to blood in terms of biochemical compounds, it is suggested that saliva may possess similar radicals scavenging ability as that of blood samples.[15,16] Strong correlation has been reported between plasma and salivary antioxidant changes in relation to physical activity.[17] However, enzymatic antioxidants including POD, SOD, and CAT have maximum concentration and are the most important.[18]

Antioxidant defense system is not able to entirely prevent oxidative stress in the strenuous exercise-training situation. The role of the antioxidant supplementation is, therefore, very important in this condition. It has been shown that garlic could eliminate hydroxyl free radicals due to the presence of thiol derivatives, allicin, and s-alyl-cyctein.[19,20] Many studies on the effect of exogenous antioxidants including vitamins E, A, and C have been done with conflicting results in most cases.[9,21]. However, the antioxidant role of garlic as a supplement on the indicators of aerobic exercise-induced oxidative stress has received less attention in the sport science area.[20,22] Williams et al.[23] showed that garlic can counteract the undesirable effects of oxidative stress induced by disease. It was found that garlic decreased indicators of cell membrane damage, malondialdehyde and creatine kinase, and it also increased the serum total antioxidant capacity (TAC).[24] Al-Numair[19] studied the antioxidant effects of garlic extract (*Allium sativum* L.) on rats and the findings showed that garlic extract consumption lead to significant increase in TAC and activity of some enzymes, such as SOD and GPX.[19]

Su et al.[22] studied the antioxidative effects of allicine, a component of garlic, in athletic men and women (18–20 years). In this study, subjects consumed 80 mg of the allicin supplement 14 days prior and 2 days after exercise testing (Downhill treadmill run). The findings indicated that in comparison to control group, the rate of plasma creatine kinase, interleukin 6 was decreased, and basal antioxidative capacity increased in supplement group, and the effect remained 48 h after exercise. However, there was no difference in the amount of enzyme SOD in both groups.[22] In a study, Morihara et al. examined garlic extract consumption along with moderate aerobic training on rats. The findings indicated that garlic consumption increased the activity of enzymes SOD and succinate dehydrogenase.[20] Koseoglu et al.[25] investigated the effect of long-term (30 days), short-term (15 days), and single sessions (3 h prior to taking the blood sample) of garlic on TAC and reported that TAC was increased in long-term and short-term supplementary situations.[25]

Considering that body cells are prone to free radicals attack, performance of the body's antioxidant defense system to deal with free radicals and ROS is very important. Therefore, the aim of this study was to evaluate the effect of short-term garlic supplementation on changes in salivary antioxidants in male athletes after a single bout of exhaustive exercise.

2. Materials and methods

2.1. *Materials*

Garlic extract capsules were obtained from Nature Made Company (USA) with sanitary justification from United States pharmacopeia (USP). SOD, POD, CAT, and their appropriate substrates were purchased from Merck and used as supplied. All chemicals and solvent were also obtained from Merck and used without further modification.

2.2. *Methods*

In a quasi-experimental and double blind study, 16 healthy young male athletes volunteered to participate as subjects. The subjects were university students who did not consume any supplements. Each subject received a verbal and written description of the study, procedures, and some points that they should observe. They then signed an informed clinical-sport

Table 1 – Physical and physiological characteristics of the subjects.

Parameters	Placebo	Supplement
Age (years)	24.7 ± 2.4	24.6 ± 2.1
Height (cm)	176 ± 7.5	175 ± 8
Weight (kg)	69.6 ± 6.9	67.65 ± 4.1
Body fat (%)	14.1 ± 1.7	12.8 ± 2.4
Body mass index (kg m^{-2})	22.4 ± 1.3	21.8 ± 1.8
Basal heart rate (beats min^{-1})	61.4 ± 1.7	60.2 ± 2.2
VO_2max (ml kg^{-1} min^{-1})	50.3 ± 2.5	52.1 ± 1.3

Values are given as mean ± SD.

and consent form. The study protocol was approved by the Moral Committee of the Department of Sport Sciences, University of Guilan. Regarding the study plan scheduling, 1 week prior to the main test (first visit), anthropometric and physiologic measurements (weight, height, skinfolds thickness, body mass index, and VO_2max) were performed (Table 1).

The subjects completed their food diary 72 h prior to the main test. They were asked to change their regimen and also avoid any exercise 3 days prior to the main test. Daily dietary intake was analyzed by "Nutritionist IV diet analysis" software and mean daily calorie, protein, carbohydrate, and fat intake were computed.

All subjects were referred to the exercise physiology lab before the supplement consumption to exhaustive test. The subjects were told to have their lunch at least 2 h before the test along with 500 ml of water (to equal hydration), brush their teeth, and wash their mouth with distilled water after lunch. Before the exhaustive aerobic test, the subjects rested on a comfortable chair for 15 min and pretest saliva sample was collected during the final 5 min. The subjects performed, then, a treadmill run after general warm up with speed of 8.05 km/h (5 mile/h) and gradient of 0%. The gradient of treadmill increased 2.5% after 3 min and was, then, increased 2.5% every 2 min gradually.[26] Second saliva samples were obtained immediately after the test and third sample was collected 1 h after test. The subjects did not eat and drink anything between sampling. Maximal oxygen consumption was calculated indirectly and with the following equation.

$$VO_2\text{max}\ (\text{ml}\ \text{kg}^{-1}\ \text{min}^{-1}) = (\text{total time} \times 1.444) + 14.99$$

After first test, the subjects were divided randomly and double blinded into garlic extract group (Group I, $n = 8$) and placebo (Group II, $n = 8$). After 24 h, they consumed 2 capsules of supplement (700 mg of garlic extract every day) or placebo (700 mg of scorched dextrose every day).[27] The placebo group consumed dextrose capsules and these capsules were scorched with garlic powder in the laboratory and the presence of garlic was indiscoverable. The subjects contributed to exhaustive aerobic test after finishing the supplementary period. Fourth, fifth, and sixth samples were obtained using same method and the samples were kept at −60 °C for final analysis.

2.3. *Determination of peroxidase activity*

0.002 M 4-amino antipyrine, 0.0010 M hydrogen peroxide, 0.3 M phosphate buffer, and 0.15 M phenol were used to measure peroxidase activity at 25 °C. A typical reaction mixture contained 495 μl of 4-amino antipyrine, 495 μl of hydrogen peroxide, and 10 μl of phosphate buffer with pH 7.0. The course of enzymatic activity was followed by spectrophotometric method on an UV-visible spectrophotometer (Ultrospec 3000 UV/Vis, Pharmacia Biotech, Sweden). The change in absorption at 510 nm (ΔA/min) was recorded.[26]

2.4. *Determination of SOD activity*

SOD activity was obtained by a method described previosly[28] using Ultrospec 3000 UV/Vis spectrophotometer. In a typical 1 ml reaction mixture, 0.1 mM ethylene diamine tetraacetic acid (EDTA), 50 mM phosphate buffer, 75 μM nitro blue tetrazolium (NBT), and 0.21 mM riboflavin were mixed with 400 μl of saliva sample. The reaction mixture was kept under a fluorescent light for 15 min with moderate shaking. The absorption of each sample was then recorded at 560 nm.

2.5. *Determination of CAT activity*

This was a modification of the method described by 50 M phosphate buffer, pH 7.0 was mixed with 20 mM hydrogen peroxide for measurement CAT activity. In the sample quartz cell, 460 μl of phosphate buffer was mixed with 40 μl of saliva samples, while the blank cell contained 500 μl of pure phosphate buffer. The course of reaction was followed kinetically at 240 nm for 2 min at every 10 s.[28]

2.6. *Statistical analysis*

The normal distribution of the variables was checked by the Kolmogorov–Smirnov (K-S) test. Description statistics was used for means and variances calculation. Also, the two-way repeated measure of ANOVA followed by post hoc Bonferroni test was used to evaluate antioxidation differences at pre- and postsupplement consumption periods after exhaustive aerobic activity. The results were expressed as mean ± SD. Significance for all analyses was set at $P \leq 0.05$ and the analyses were conducted using the Statistical Package for Social Sciences (SPSS v. 20®, Inc. Chicago, IL).

3. Results

Daily dietary intake during 3 days before activity of subjects was computed by N4 Program and the results are presented in Table 2. Study findings indicated that there is no significant difference between groups in relation to daily dietary intake ($P > 0.05$).

Figs. 1–3 indicate changes in CAT, SOD, and POD, respectively.

POD was changed in inside-group factor with same P values ($P = 0.0001$), time and group interaction ($P = 0.0001$, 0.026 and 0.028), and between-group factor (supplement and placebo) ($P = 0.021$, 0.044 and 0.011), respectively. Therefore, garlic extract consumption led to significant increase in salivary activity of SOD, CAT, and POD after a single bout of exhaustive aerobic exercise.

Table 2 – Dietary intake assessed during the 3 days before each exercise session.

Variables	Group	Value
Calorie intake (cal)	I	2310 ± 190.1
	II	2379 ± 287.4
Carbohydrate (g)	I	305.32 ± 31.7
	II	289.68 ± 59.17
Fat (g)	I	87.54 ± 3.03
	II	92.2 ± 1.9
Protein (g)	I	122.06 ± 12.24
	II	115.6 ± 16.25
Vitamin E (mg)	I	16.4 ± 2.85
	II	17.3 ± 1.39
Vitamin C (mg)	I	84.56 ± 5.7
	II	85.68 ± 3.42

I, garlic extract group; II, placebo group; values are given as mean ± SD.

4. Discussion

Production of ROS leads to increase in oxygen usage followed by electron leakage from mitochondrial electron transport chain. This will cause tissue damage, activation of inflammatory cells, auto-oxidation of catecholamines, xanthine dehydrogenase pathway, and auto-oxidation of oxyhemoglobin than methemoglobin. Muscle cell hypoxia and hydrogen superoxide transformation to hydroxyl radical by lactic acid are the final results.[7] Thus, any activity that increases oxygen consumption can lead to continuous increase in free radicals and oxidative stress.[6] Insufficient antioxidant defense system may increase oxidative stress, which is accompanied by metabolic changes and leading to poor performance in athletes.[8] Therefore, intense exercise leads to excessive formation of ROS in plasma and in white blood cells.

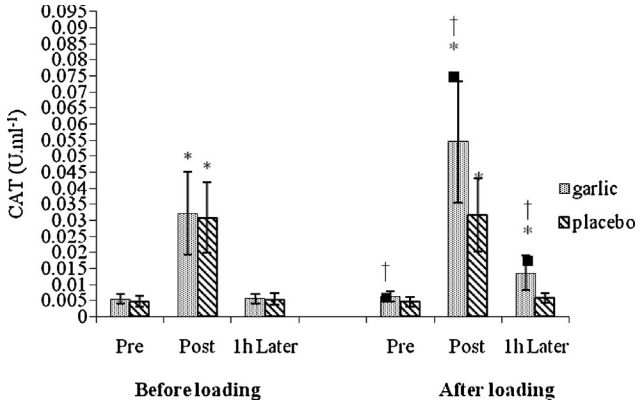

Fig. 1 – Changes in CAT concentration during pre- and postsupplement consumption periods at before, immediately, and 1 h after test in supplement and placebo groups. * Significantly different in comparison with pre-exercise ($P \leq 0.05$). † Significantly different in comparison with before supplementation ($P \leq 0.05$). ■ Significantly different in comparison with placebo group ($P \leq 0.05$).

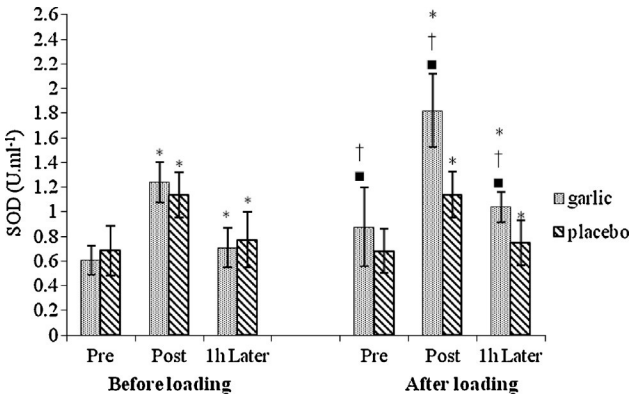

Fig. 2 – Changes in SOD concentration during pre- and postsupplement consumption periods at before, immediately, and 1 h after test in supplement and placebo groups. * Significantly different in comparison with pre-exercise ($P \leq 0.05$). † Significantly different in comparison with before supplementation ($P \leq 0.05$). ■ Significantly different in comparison with placebo group ($P \leq 0.05$).

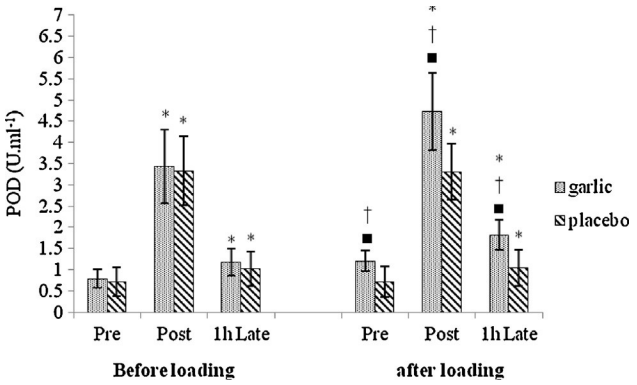

Fig. 3 – Changes in POD concentration during pre- and postsupplement consumption periods at before, immediately, and 1 h after test in supplement and placebo groups. * Significantly different in comparison with pre-exercise ($P \leq 0.05$). † Significantly different in comparison with before supplementation ($P \leq 0.05$). ■ Significantly different in comparison with placebo group ($P \leq 0.05$).

Therefore, it is directly related to muscular damage and oxidative stress.

Application of necessary measures to reduce probable damages caused by ROS is particularly important during intense exercises. This is possible by reducing the exercise intensity to suppress production of ROS or by strengthening the body's antioxidant defense system. Considering that athletes should perform intense training, it seems likely that strengthening the body's antioxidant defense system through supplementation could be a preferable strategy. As supplementation with antioxidants is still controversial among many researchers, in the present study we investigated the effect of supplementation with garlic on the activity of three, most important, salivary antioxidant enzymes. The results showed

significant increase in the activity of SOD, CAT, and POD in the case of group I who consumed garlic extract in addition to exercising.

The effect of moderate antioxidant supplementation has been studied using *lemon verbena* extract in healthy male volunteers who performed a 90-min running eccentric exercise protocol for 21 days.[29] Antioxidant enzymes activities and oxidative stress markers were then measured in neutrophils. It was shown that intense running exercise for 21 days induced antioxidant response in neutrophils of trained male through the increasing activity of CAT, GPX, and glutathione reductase. These results reported in 2011 support the findings we observed in our research.

Recently, the effect of long-term supplementation with β-hydroxy β-methylbutyrate free acid on muscle mass and its strength has been reported.[30] However, the oxidative damage that may be induced due to long duration of exercise is not considered in that study. Scavenging free radicals is a suitable way to prevent the oxidative damage by high free radical production.

The findings of our present study indicated that, a relatively short-term, 14 days, consumption of garlic extract can induce favorable effects on the important antioxidant enzymes CAT, SOD, and POD.[31] Supportive to our results, it has been suggested that garlic can improve TAC by increasing intracellular antioxidants, such as glutathione, uric acid, and bilirubin, as well as enhancing the expression of intracellular antioxidant enzymes SOD, CAT, and GPX.[6,20,32] It has also been found that garlic reduced the activity of tumor necrosis factor-α (TNF-α) and disables nuclear factor-kappaβ (NF-kβ) causing a marked decrease in oxidative damages. Okada et al.[33] studied the antioxidative mechanism of allicin as one of the main thiosulfinates in garlic and expressed that antioxidative property of allicin is due to inhibition of transport chain of peroxy radicals and allylic peroxides transition from substances and compounds.[33]

CAT and POD react with hydrogen peroxide and convert it to H_2O and O_2 in response to the increase in free radicals caused by aerobic exercise. SOD could also neutralize the superoxide radicals leading to reduced ROS damage. Conversion of superoxide radicals by SOD is one of the important mechanisms of resistance against stress. So, probably the reason for the sudden increase in the activity of this enzyme is body's biological response to overproduction of free radicals.[34] The protective action of CAT is through quick and direct cleanup of hydrogen peroxide, as well as its cooperation with SOD. So that CAT protects SOD against inactivation, which may be caused by increased levels of hydrogen peroxide.[2,30]

A detailed analysis of our results revealed that immediately and 1 h after exercise, the activity of all three enzymes (CAT, POD, and SOD) increased more significantly ($P < 0.05$) as compared to before exercise ($P > 0.05$). We have previously examined the salivary antioxidant changes in male athletes after intense exercise.[28] The results of that study support our present finding in terms of TAC in saliva. We had found that uric acid concentration and activity of SOD, CAT, and POD increased significantly after exercise.[28] In support of the present research, Cavas et al.[35] have also shown that after judo exercise, the activity of salivary SOD, CAT, and GPX was significantly increased compared to before exercise.[35] They, however, did not use any supplement during their exercise period and they used blood samples as diagnostic body fluid. One of the highlights of the present study is the use of saliva instead of blood. Saliva is the first line of defense against consumption of any damaging species entering gastrointestinal tract. Its collection is easy and noninvasive in character and because of its special biochemical composition, it can be used as alternative to invasive sampling of blood.

The results of this study were inconsistent with those reported by Faruk Ugras[36] and Tsai et al.[37] who did not find significant increase in antioxidant activity after exercise. The inconsistency in results may be due to differences in the exercise protocol used in the mentioned study. For example, running on a treadmill to exhaustion or weight loss in taekwondo, with different metabolic needs, may have different ROS generating systems.[38,39] The intensity of physical activity, type of sampling (saliva vs plasma), and the method of assessment are also determinants in the study.

The present results indicate a significant increase in salivary enzymes SOD, POD, and CAT activities after exhaustive aerobic exercise in pre- and postsupplementation period. Consistent with these results, Al-Numair[19] investigated the antioxidant effect of garlic extract (*A. sativum* L.) on mice. They reported that garlic extract consumption led to significant increase in TAC, SOD, and GPX activity in mice.[25] Koseoglu et al.[25] investigated the effect of garlic supplementation on serum TAC and showed that serum TAC of healthy men increased in long- and short-term supplementation.[24] The results of Dhawan et al.[24] and Durak et al.[39] revealed that garlic with antioxidative effects can react against undesirable effect of oxidative stress caused by disease. Garlic can decrease the indicator of cell membrane damage and also, it can increase serum antioxidative capacity (TAC).[24,29]

Some researchers have reported less antioxidant alternations due to food supplements.[23,39] The inconsistency in results could be due to differences in the type of subjects, as the subjects of mentioned study were cardiovascular patients and with atherosclerosis (45–70 years). Age and disease can affect antioxidant responses, so that basal antioxidative power is reduced when the age and severity of disease is higher.[23] The results of SOD activity in our study differed from what was reported by Su et al.[22] The reason for this inconsistency is associated with subject's physical fitness, type of exercise, and measurement method.

Long-term supplementation with natural antioxidants has been studied using green tea extract (GTE) for 4 weeks.[40] The study was conducted using GTE together with strength training in a randomized, double blind design. A number of oxidative stress markers in blood were then investigated. Jówko et al.[40] then showed that supplementation with GTE enhanced plasma total polyphenols at rest and 5 min after the muscular endurance test. They also found that GTE contributed to the rise of resting total antioxidant status in plasma. Although the studies on natural antioxidant supplementation are mostly related to long-term use, their results support our finding in most cases.

Long-term overloaded training combined with short-term supplementation with mixture of selenium, retinol, ascorbic acid, and alpha-tocopherol has been reported to reduce oxidative damage by modulating antioxidant potential.[41–44]

These types of research led to the conclusion that during tapering training (TT), antioxidant supplementation at nutritional doses could affect exercise-induced oxidative stress in favor of reducing possible damages by ROS.

5. Conclusions

Based on the results obtained in this study, it can be concluded that short-term (700 mg of garlic extract every day) consumption of garlic extract supplement possibly can neutralize the harmful effects of free radicals due to exhaustive aerobic activities. The considerable increase in antioxidative activity of SOD, peroxidase, and CAT is an indicator of higher antioxidant action in salivary fluid of subjects who were supplemented with garlic extract. This type of high antioxidant power could, therefore, compensate the unfavorable result of oxidative damages in athletes.

On the other hand, valuable findings in this study suggest that supplementation with moderate levels of an antioxidant, such as garlic extract, could significantly reduce exercise-induced oxidative damage of proteins and lipids in salivary fluid through the action of antioxidant enzymes, CAT, SOD, and POD. It seems likely that a supplement containing an appropriate combination of antioxidants may induce a more favorable result in this aspect.

Conflicts of interest

The authors have none to declare.

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