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Effect of Spirulina supplementation on DNA damage after exhaustive exercise in athlete men

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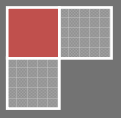
ABSTRACT

This study was performed to determine Effect of short-term spirulina supplementation on DNA damage after exhaustive exercise in athlete men. Twenty male athletes (aged 21.06±1.02years, weight 65.12±1.83 kg and height 172.25±1.63 cm and BMI 21.73±0.73 kg/m2) in a randomized and double-blind design were allocated in two equal supplement and placebo groups (3g/day spirulina for 14 days). After supplementation, all participants were participated in bruce test. The blood samples were taken in three phases (before and after the supplementation and after the exercise). This experiment used single cell gel electrophoresis including modified comet assay (with FPG and ENDO III enzymes) to detect the oxidative DNA damage induced by exhaustive exercise in athlete blood cells, and the product 8-hydroxy- 2-deoxyguanosine (8-OHdG) of oxdation of DNA, also, the level of MDA, BUN and CK in athlete plasma were observed. The normal data (Mean ± SD) were analyzed by repeated measure ANOVA, Tukey and independent t-test ($P \leq 0.05$). The results showed that a 14-day spirulina supplementation hadn't significant effect on the damage index of modified comet assay (with FPG and ENDO III enzymes) and the level of 8-OHdG and also the level of MDA, BUN and CK in plasma at rest status ($P > 0.05$). However, exercise-induced decrease of 8-OHdG and the value of comet assay and the plasma level of MDA, BUN and CK in the spirulina group were significantly more in comparison with those in the placebo group ($P < 0.01$). Result of the study indicates that 14-day spirulina supplementation can reduce the production of lipid oxidation and oxidative DNA damage during exhaustive exercise.

KEYWORDS

Spirulina supplementation; DNA damage; Single cell gel electrophoresis (SCGE); exhaustive exercise.



INTRODUCTION

It is known that both acute aerobic and anaerobic exercise has the potential to result in increased free radical production, if the increase in reactive oxygen species (ROS) exceed the antioxidant defense system present, thereby resulting in oxidative damage to specific biomolecules and the extent of oxidative damage mostly depend on both intensity and duration[1]. Oxidative damage of biomolecules such as DNA and lipids has been implicated in the modification of aging and degenerative diseases and reactive oxygen species (ROS) play an important part as mediators of tissue injury and inflammation after strenuous exercise[2]. Aside from the measurement of 8-OHdG[3], assessment of DNA damage has also been performed using the single cell gel electrophoresis assay (Comet assay) which detects DNA damage with high sensitivity[2]. It has been experimentally confirmed antioxidant supplements could prevent or alleviate the DNA damage. Recently, different studies indicated that natural antioxidants contained in vegetables and fruits (flavonoids, carotenoids) may be useful in preventing deleterious consequences of oxidative stress[4], so antioxidant supplements are commonly used by athletes as dietary nutrition to counteract the oxidative stress of exercise to increase exercise performance. A study in 2007 found that spirulina was used as a dietary supplement to improve the antioxidant potential of many geriatric patients who had taken it for 16 weeks and the plasma of these patients showed a measured increased level of total antioxidant status[5]. Spirulina supplementation significantly reduced the increased lipid peroxidation level in HCD-fed rabbits[6]. Some studies have reported that Spirulina supplementation induces an obvious increase in exercise performance, the mechanism of which may be through an increase in b- oxidation pathway rate and increases the reducing levels of glutathione[7].

Spirulina contains vitamin B1 (thiamine), B2 (riboflavin), B3 (nicotinamide), B6 (pyridoxine), B9 (folic acid), vitamin C, vitamin D, vitamin A, and vitamin E[8]. So spirulina can also acts as vitamin E which could prevent the DNA damage of toxic effect to the body, and prevent or reduce the form of free radical during human exposure to ionizing radiation, smoking, food additives in the daily environment[9]. However, the current studies about the effect of Spirulina on preventing DNA damage after exhaustive exercise are still rarely reported. Whether exhaustive exercise does increase the need for additional antioxidants in the diet is not clear. This experiment used single cell gel electrophoresis including modified comet assay (with FPG and ENDO III enzymes) to detect the oxidative DNA damage induced by exhaustive exercise in athlete blood cells, and the product 8-hydroxy- 2-deoxyguanosine (8-OHdG) of oxdation of DNA, also, the protective effect of Spirulina on athlete blood cells were observed, all of which were to provide a reference basis on how to supplement Spirulina in human body. Furthermore, it was aimed to observe the effect of Spirulina to reduce the oxidative stress and whether could it enhance the DNA repair capacity to increase exercise ability.

MATERIALS AND METHODS

Subjects

16 athlete male students majored in physical education of Minnan Normal University (aged 21.06±1.02years, weight 65.12±1.83 kg and height 172.25±1.63 cm and BMI 21.73±0.73 kg/m2), to participate in this research, as the samples were selected voluntarily and with their consent. Subjects were selected according to the following criteria: nonsmoking and apparently healthy. Furthermore, all selected subjects were not taking any antioxidant supplements (such as vitamin A, C, or E) 2 month before and during the study; they were asked to follow a rigorously standardized basal diet, avoid of spirulina products. Volunteer subjects randomly replaced in two groups of receiver of spirulina supplements (3g daily for two meals a day for fourteen days) and placebo (dextrose capsule). For control of subjects Nutrition, dietary questionnaire of 24-hour retention was used.

Exercise protocal

First day of study, height, weight and percentage body fat was measured in all subjects. Initial blood sample, at baseline, before starting supplementation, were taken from the Antecubital vein from all the participants. Second samples were taken after completion of 14-day period of supplementation and before Bruce test. During the Bruce test, the VO2max and heart rate (HR) were recorded. The VO2max situation was accepted if the oxygen uptake reached a plateau, the plasma lactate concentration was above 8 mmol/l, or the respiratory exchange rate was above 1.15. After the Bruce test, third samples were taken from subjects. At each stage of depletion, Peripheral blood samples were collected at different time points from patients and control individuals, at rest, early in the morning, into two tubes with anticoagulant. One aliquot was used for the comet assay and the other aliquot to obtain blood plasma for the remaining analysis. All measurements were done in same temperature, humidity, ventilation and lighting. In addition, subjects, 48 hours before the test, don't do any heavy physical activity, and their meal before the test was similar.

Alkaline comet assay

The alkaline comet assay was done as described by Godard et al.[10]. Fully-frosted clean microscope slides were covered with 1% normal melting point (NMP) agarose and allowed to polymerize at room temperature to allow agarose to dry. After solidification, the gel was scraped off from the slide. The slides were further coated with 0.6% NMP agarose. When this layer had solidified a second layer containing the whole blood sample (0.5μL) mixed with 0.5% low melting point (LMP) agarose was placed on the slides. After 10 min of solidification on ice, slides were covered with 0.5% of LMP agarose. An amount of 100 μL of this agarose cell suspension was layered on the top of the second layer. Finally, the fourth layer of 0.5% low melting point (LMP) agarose was added to cover the third layer and allowed to solidify for 10 min at 4ºC.

Afterwards the slides were immersed for one hour in ice-cold freshly prepared lysis solution (2,5M NaCl, 100 mM Na_2EDTA, 1% Na-sarcosine, 1% 10 mM Tris–HCl, pH 10 with Triton X-100 and 10% DMSO added fresh to lyse cells and allow DNA unfolding). After lysis, slides were placed in the freshly prepared electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0) to remove salts. The slides were set in this alkaline buffer for 10 min to allow DNA unwinding and expression to alkali labile sites. Denaturation and electrophoresis was performed at 4 ºC under dim light at 25 V (300 mA). After electrophoresis, the slides were washed three times at 5 min intervals with buffer (1% Triton X-100, 10%DMSO) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 μg/ml) for 10 min and covered with a cover slip. Slides were stored at 4ºC in humidified sealed containers until analysis. To prevent additional DNA damage, handling with blood samples and steps included in the preparation of slides for the comet analysis were conducted under yellow light or in the dark.

Slides were examined at 100× magnification on an Olympus fluorescence microscope (Olympus Optical Co, Ltd, Tokyo) with excitation at 520 nm green barrier filter. For DNA damage evaluation, 100 cells per sample were analyzed by optical microscopy at 100x magnification. The cells were visually scored by measuring the DNAmigration length and the amount of DNA in the tail into five classes, from undamaged −0 to maximally damaged −4, and a damage index (DI) value was calculated for each sample[11]. Damage index, thus, ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage:100 cells × 4).

Detection of Oxidized Bases

The comet assay was modified by using specific enzymes to expose oxidative damage. The enzyme formamidopyrimidine DNA glycosylase(FPG) recognizes the common oxidized purine 8-oxo-7,8-dihydroguanine and ring-opened purines[12], whereas endonuclease III (ENDO III) converts oxidized pyrimidines to strand breaks[13]. After lysis, the slides were washed for 5 minutes each in enzyme buffer (40mM HEPES-KOH, 1MKCl, 5mMEDTA, 2.5mg/mL bovine serumalbumin fraction V-BSA, and pH 8.0). The suspension was added to the slide, covered with coverslip, and incubated for 45 (ENDO III) and 30 minutes (FPG) at 37◦C. Subsequent steps were the same as in the alkaline version of comet assay.

Measurement of plasma 8-OHG

Determination of plasma 8-OHG levels was performed using the method of Farhadi et al[14]. For the measurement of plasma 8-OHG, 1 ml plasma was spiked with 1,000 cpm [14C]-OHG. Plasma protein was precipitated by the addition of an equal volume of acetonitrile, and precipitated protein was separated by centrifugation at 3,000g for 15 min at 4°C. Supernatant was transferred to a new tube and mixed with eight volumes of water. The resulting sample was applied to the preconditioned C18/OH solid-phase extraction column. The solidphase extraction column was washed with 5 ml of 50 mmol/l KH2PO4 buffer (pH 7.5), and then retained. Compounds were eluted with 3 ml of 15% methanol in the same buffer. The elute was loaded into the immunoaffinity column prepared with monoclonal antibody for 8-OHG. Purified 8-OHG was dissolved in 50 ml water and injected into an HPLC device equipped with a Beckman Ultrasphere ODS column (5 cm, 4.6 cm, 25cm) and an electrochemical detector. The height of the 8-OHG peak and the total radioactivity of the elute were measured. The height of the peak was used to determine the total amount of 8-OHG injected, which was the sum of the plasma 8-OHG and [14C]-OHG added. The amount of 8-OHG was determined by subtracting the amount of [14C]-OHG injected from the total amount of 8-OHG injected. The amount of [14C]-OHG injected was determined from the calibration curve of the peak height of [14C]-OHG. The radioactivity of the elute was used to determine the amount of loss of 8-OHG during the purification procedure using the immunoaffinity column.

Estimation of Plasma MDA, BUN and CK

Aliquots of whole blood were taken immediately for the comet assay analysis. Remaining blood was centrifuged at 2500×g for 10 min; Plasma was then aliquoted to cryotubes for various assays. The process of lipid peroxidation results in the formation of malondialdehyde (MDA). Lipid peroxidation was determined indirectly by measuring MDA formed by reacting with thiobarbituric acid (TBA) to give a red species having a maximum at 532 nm. MDAwas determined spectrophotometrically according to the method of the Nanjing Jiancheng Bioengineering Institute(China) with a spectrometer. Plasma creatine kinase (CK)activity and blood urea nitrogen(BUN) were measured for evaluating the cell membrane injury by a model 7170A automatic analyser (Hitachi) with commercial assay reagents (Amresco).

Statistical analysis

The data are presented as means ± SD of independent experiments. They were analyzed using the software of statistical package for the social sciences (SPSS) version 16.0 for Windows. The statistical difference between groups was determined with repeated measure ANOVA followed by Dunnett's test (two-sided) as multiple comparisons. The minimum level of significance was considered to be $P<0.05$.

RESULTS

The general characteristics of the placebo group and spirulina group are shown in TABLE 1. T-test results showed no significant differences between the variables of height, weight, age, BMI and percentage body fat of two groups' subjects that indicated to be homogeneous both groups in these variables.

TABLE 1: Personal Characteristics of spirulina and placebo groups

Variable	N	Placebo Group	Spirulina Group
Age(years)	8	21.37±1.06	20.75±1.03
Height(cm)	8	172.3±1.66	172.25±1.83
Weight(kg)	8	64.87±2.10	65.37±1.83
BMI(kg/m2)	8	21.78±0.98	21.69±0.51
Percentage body fat (%)	8	13.98±2.43	14.03±1.38

TABLE 2: Comet assay results between placebo group and spirulina group in different phases

Variable	Group	N	baseline	after supplementation	after exhaustive test
Damage index alkaline comet assay	placebo	8	31.71±24.63	31.50±21.15	57.62±29.15 *#
	spirulina	8	34.26±24.82	28.62±16.97	42.12±25.64
Damage index FPG comet assay	placebo	8	39.16±26.37	40.57±24.62	84.77±27.96**##
	spirulina	8	38.41±18.44	41.49±16.99	47.11±22.06
Damage index ENDO III comet assay	placebo	8	41.75±17.31	39.62±18.29	85.22±20.17**##
	spirulina	8	42.12±23.11	40.25±24.35	53.11±19.27

All the data was expressed by x ±s, *P<0.05, **P<0.01, Significant difference between garlic and placebo groups; # P<0.05, ## P<0.01, Significant difference after supplementation and after exercise protocol

The comet assay results are shown in TABLE 2. DNA damage is visualized at the individual cell level as an increased migration of genetic material (‘‘comet tail’’) from the nucleus (‘‘comet head’’). The basal DI in the alkaline comet assay that detects DNA single- and double-strand breaks and alkalilabile sites, the results of which showed that the higher this values, the greater the damage that has occurred to the nuclear DNA. The value of DI in the alkaline comet assay did not show sinificant diffenence between placebo group and spirulina groups at rest(P>0.05). After supplementation, the results of DI in the alkaline comet assay still did not have obvious sinificance compered with the value of baseline for both two groups, at the same time, there was no obvious sinificance between placebo group and spirulina groups(P>0.05). However, the value of DI in the alkaline comet assay showed that spirulina and placebo groups have significant difference after exhaustive exercise (bruce test) (P<0.05). Also, the value of DI in modified comet assay (with FPG and ENDO III enzymes) showed significantly higher damage index in placebo group compared with spirulina group after exhaustive exercise(P<0.01), and there was no significant difference between groups at baseline and after supplementation (P>0.05).

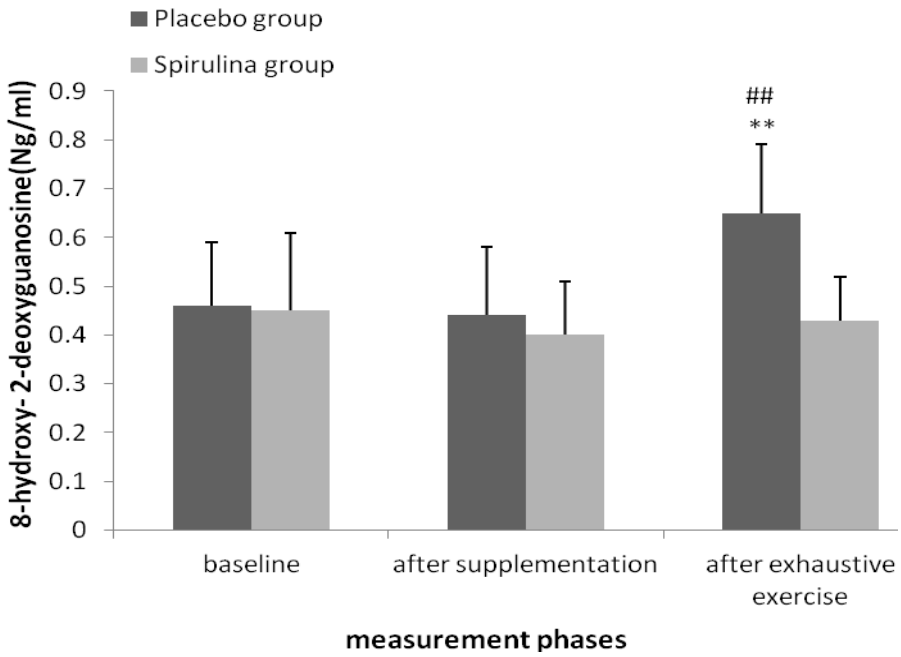


Figure 1: Plasma 8-hydroxy- 2-deoxyguanosine in baseline and after supplementation and exhaustive exercise of spirulina and placebo groups

All the data was expressed by x ±s, *P<0.05, **P<0.01, Significant difference between garlic and placebo groups; # P<0.05, ## P<0.01, Significant difference after supplementation and after exercise protocol

TABLE 3: The changing of BUN, CK and MDA on plasma in different group

Phases	Group	N	BUN(mmol/L)	CK(IU/L)	MDA(nmol/mL)
Baseline	placebo	8	5.81±0.87	203.63±99.72	4.83±0.95
	spirulina	8	5.78±0.91	201.62±87.53	4.93±0.98
after supplementation	placebo	8	5.82±0.84	204.25±92.26	4.87±1.05
	spirulina	8	5.77±0.74	198.50±82.71	4.84±0.92
after exhaustive exercise	placebo	8	6.49±1.25*$^{\#}$	256.87±112.32*$^{\#}$	7.21±1.16**$^{\#\#\#}$
	spirulina	8	5.67±0.78	218.75±103.53	5.60±1.05$^{\#}$

All the data was expressed by x ±s, *P<0.05, **P<0.01, ***P<0.001,Significant difference between garlic and placebo groups; $^{\#}$ P<0.05, $^{\#\#}$ P<0.01,
$^{\#\#\#}$ P<0.001,Significant difference after supplementation and after exercise protocol

As observed in Figure 1, the product 8-hydroxy- 2-deoxyguanosine (8-OHdG) has been measured as an index of exercise induced oxidation of DNA[14], and the level of plasma 8-OHdG of repeated measure ANOVA for any groups showed that significant difference in effect of measurement phases in spirulina group (P≥0.05), but, significant difference showed in placebo group (P≤0.01). The results of Tukey post-hoc test for placebo group showed significant difference in before and after bruce test (P≤0.01). Also, statistically significant differences (P≤0.01) in the level of plasma 8-OHdG were found between placebo group and spirulina groups through independent t-test.

From the TABLE3, it showed that the level of BUN and CK did not have significant differences between placebo group and spirulina groups (P>0.05) at baseline and after supplementation. However, both the level of BUN and CK increased significantly after exhaustive exercise compared with the value of BUN and CK at baseline and after supplementation (P<0.05). For the level of MDA, there were no significant differences between groups (P>0.05) at baseline and after supplementation, but, the value of MDA in placebo group increased obviously after exhaustive exercise compared with the value of MDA at baseline and after supplementation (P<0.001), as well as the MDA level in spirulina group significantly increased after exhaustive exercise compared with baseline and supplementation (P<0.05). Also, there were significant difference between placebo group and spirulina groups after exhaustive exercise (P<0.01).

DISCUSSION

It is well known that most of athletes have to run to exhaustion to achieve wonderful exercise performance, and it is clear that the consequences develop in the days following severe exercise are soreness and stiffness, which would give the athlete a big trifle to begin a new training.[15]. Moreover, during exercise there would be a 10-40-fold increase in oxygen consumption compared to the resting state, specially, endurance exercise may contribute to a two-to three- fold increase in the free radical concentrations of the muscle and the liver[16]. Reactive oxygen species (ROS) cause lipid peroxidation and oxidation of some specific proteins, thus affecting many intra- and intercellular systems[17]. DNA damage are physical abnormalities in the DNA, such as single- and double-strand breaks and oxidative DNA damage might result in diseases[18].

Comet assay has been widely used in various studies to detect the DNA damage connected with kinds of diseases due to its rapid, simple, and sensitive technique for measuring DNA breaks and repair in single cells[19]. DNA damages can be recognized by enzymes, and, thus, they can be correctly repaired if redundant information, such as the undamaged sequence in the complementary DNA strand or in a homologous chromosome, is available for copying[20]. Moreover, DNA damaging agents can damage other biomolecules such as proteins, carbohydrates, lipids, and RNA. Generally, DNA damage is the leading basis in the carcinogenic process, however DNA can be repaired, for the body has some repair mechanism after injury, so as to avoid the occurrence of tumor [21][22][23]. Blood mononuclear cell DNA oxidative damage is found to increase in human body after intense exercise[2]. It was also found that the DNA of skeletal muscle cells, lymphocytes and blood cells appeared damage to some extent[24]. In the present study, the SCGE technique was used to detect DNA damage in athlete blood cell,the results showed that the value of damage index in the alkaline comet assay of placebo groups were significantly increased post exhaustive exercise compared with rest status and spirulina group (P<0.05), suggesting that DNA of athlete blood cells appeared serious injury, this result supported previous reports[25][26][27].

Most damage to bases in DNA is repaired by the base excision repair pathway. Formamidopyrimidine DNA glycosylase (FPG) is a base excision repair enzyme which recognizes and removes a wide range of oxidized purines from correspondingly damaged DNA[28]. Also, endonuclease III (Endo III) is major repair enzyme for pyrimidine lesions formed by reactive oxygen species[29]. In this study, the value of the value of DI in modified comet assay (with FPG and ENDO III enzymes) increased severely after exhaustive exercise in placebo group compared to spirulina group (P<0.01). It was suggested that there was severe oxidative DNA damage produced in athlete blood cell during exhaustive exercise.

Cumulative oxidative DNA damage have a significant effect of the impairment on normal cellular repair mechanisms and one of the main etiological hypotheses linking genomic instability, mutagenesis and tumorigenesis is that of deficient cellular repair mechanisms due to extensive oxidative DNA damage and cellular injury[30]. The resultant damaged bases in DNA may be responsible for mutations that lead to carcinogenesis. One of the major forms of oxidative DNA damage,8-hydroxydeoxyguanosine (8-OH-dG) has been proposed as a key biomarker relevant to carcinogenesis[14]. It is

usually for our organisms exposed to reactive oxygen species (ROS) produced by environmental hazards, such as radiation and toxic chemicals, and also by physical activities, so it would beneficial for our health, especially for the athlete, to find out the protective supplements to reduce muscle damage.

Antioxidants work together in animal cells against toxic reactive oxygen species[24]. Spirulina is a popular nutritional supplement that is accompanied by claiMSS for antioxidant and performance-enhancing effects[31]. Spirulina has been used as a complementary dietary ingredient of feed for poultry and increasingly as a protein and vitamin supplement to aqua feeds [32]. It is well accepted that lipid peroxidation was induced by free radicals. The present study also investigates the status of lipid peroxidation. Malondialdehyde (MDA) is the product of lipid peroxidation and its level is a marker of lipid oxidation, also its content can reflect the degree of lipid peroxidation in vivo and indirectly reflect the extent of the damage of cells[33]. The results of the present experiments showed that plasma MDA activity increased significantly after acute exercise as well as DNA damage in athlete blood cell. The release of cytoplasmic enzymes including creatine kinase (CK), which was considered to be an available parameter for the working muscle injury during exercise, and CK activity was the most specific and sensitive indicator to detect and monitor for the muscle injury[34]. BUN (blood urea nitrogen) is a marker of kidney function, and it is a waste product of cell metabolism. In this study, the level of CK and BUN increased significantly after exhaustive exercise in placebo group compared with spirulina group. However, the Spirulina supplementation reduce the MDA level and DNA damage, which was suggested that DNA damage was related with tissue lipid peroxidation, and the high intensity exercise caused oxidative damage in the blood and eventually lead to DNA damage of blood cells. The fact that, in the present study, a significant level of DNA damage was detected after exhaustive running in the athlete probably shows that DNA might be a weak link in a cell's ability to tolerate oxygen free-radical attack. It is conceivable that the levels of exercise attained in our experiments could be associated with oxidative stress, and perhaps the deleterious effects associated with such stress. It is possible that a depression in the running performance of athlete could be attributed to disruption of the oxidant/antioxidant balance consequently resulting in oxidative stress.

Spirulina as an antioxidant one hand can inhibit the oxidase enzymes system, activate and protect anti-oxidase system, on the other hand, it can directly react with lively free radical, change lipid peroxides into hydroxyl resin, and it is a strong free radical scavenger[9]. The study found that oxidative DNA damage of spirulina group were significantly lower than that of placebo in athlete blood cell after exhaustive exercise, indicating that Spirulina can effectively alleviate DNA damage of blood cell caused by high intensity exercise, the reason may be related to antioxidant effect in vivo on Spirulina. The results also showed the level of CK, BUN and MDA content in spirulina group were significantly lower than those of placebo group in plasma of athlete, also, the present results indicate that short-term spirulina supplementation significantly decreased 8-hydroxy- 2-deoxyguanosine after exhaustive activity in the spirulina group compared to the placebo group, which suggesting the supplement of Spirulina can alleviate the oxidative damage effect on the blood induced by high intensity exercise, which reduce the free radicals attacking the blood nuclei and nuclear genetic material. The possible mechanism for spirulina in reducing oxidative DNA damage is that spirulina with increasing intracellular antioxidants such as bilirubin, uric acid, and serum albumin can enhance the total capacity of antioxidant.

In conclusion, the current data indicated that strenuous exercise induced elevated DNA damage due to lipid peroxidation in organisms. The evidence from the present investigation suggests that exhaustive exercise increased oxidative DNA damage as measured by modified comet assay (with FPG and ENDO III enzymes) and plasma 8-OHdG level and also short-term spirulina supplementation decreased oxidative DNA damage caused by exhaustive exercise. Spirulina supplementation inhibits lipid peroxidation and has free radical scavenging activity, which can be beneficial for the protection against oxidative stress and reduced the DNA damage. So it is beneficial for the athletes to take spirulina supplementation to relieve oxidative stress to prevent from more DNA damage.

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