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The Effect of one-week spinach and NBS superfood supplementation on interleukin-6, superoxide dismutase, and malondialdehyde levels after repeated bouts of wingate test in trained men

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Abstract

Background Consuming nutritional supplements for improving athletic performance has always been considered an ergogenic aid. However, there is limited information on the efficacy of this strategy for enhancing antioxidant capacity in response to strenuous exercise with repeated nature. Therefore, this study investigated the effect of one week of spinach and NBS superfood supplementation on superoxide dismutase, malondialdehyde, and interleukin-6 in response to repeated bouts of Wingate test.

Methods Fifteen trained men voluntarily participated in this within- participants and counter-balanced study. Participants were exposed to three different conditions including (1) seven days of raw spinach supplementation (daily dose of 70 g); (2) seven days of NBS superfood supplementation (daily dose of 10 g); and (3) the control condition. One day after the supplementation period, participants came to the lab and performed 3 bouts of 30-second Wingate test with 4-minute rest intervals. Before the supplementation and 24 h after performing the Wingate, test blood samples were taken in each condition to measure serum levels of superoxide dismutase, malondialdehyde, and interleukin-6. One week of wash-out was applied between the conditions. Two-way repeated measures ANOVA were used for statistical analysis.

Results The results showed that at post-test measurement the serum level of interleukin-6 was significantly lower under the spinach and superfood conditions ($p = 0.001, p = 0.003$, *respectively)* compared to the control. The serum level of superoxide dismutase under the spinach ($p = 0.035$) and superfood ($p = 0.01$) conditions was significantly higher compared to the control at post-test measurement. Also, at post-test measurement, the serum level of malondialdehyde was significantly lower under the spinach ($p = 0.001$) and superfood ($p = 0.017$) conditions compared to the control.

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Conclusion According to the results of the present study, it seems that seven days' supplementation of either raw spinach or NBS superfood could positively affect the inflammatory responses to repeated anaerobic all-out activities. Therefore, these supplementation strategies might be considered ergogenic aids and used by athletes before participating in repeated strenuous activities.

Keywords Nutritional supplementation, Anaerobic activity, Inflammatory response, Ergogenic aid

Background

High-intensity exercise increases reactive oxygen species (ROS) levels in skeletal muscles, resulting in inflammation, oxidative stress, and force loss [1]. ROS are highly reactive molecules and can react with biological macromolecules in cells such as carbohydrates, nucleic acids, lipids, and proteins due to the presence of unpaired electrons in their structure and disrupt their normal function [2, 3]. Intense exercise also causes oxidative stress in human blood cells and leads to lipid peroxidation, DNA damage, mitochondrial abnormalities, and protein oxidation [4, 5]. It has been shown that an acute bout of intense exercise can result in the accumulation of plasma malondialdehyde (MDA) and also pro-inflammatory cytokines such as interleukin-6 (IL-6), lasting from minutes to even 48 h after exercise [6–8]. Such molecules are considered the biomarkers of oxidative stress and might trigger unfavorable signaling cascades leading to cytotoxic condition in the body, inadequate recovery, muscle damage, and decreased physical performance [9–15]. Under such a condition, the activity of antioxidant enzymes such as superoxide dismutase (SOD) increases in order to neutralize free radicals and prevent harmful effects of ROS [16, 17].

Considering the harmful consequences of oxidative stress, applying effective strategies to cope with this condition has been a research area of interest for years [18]. Spinach is one of the most important antioxidant-containing food plants, which has a considerable amounts of nitrate, carotenoids, and flavonoids and is a good source of vitamins A, B3, and C and minerals such as potassium, calcium, magnesium, and iron [19]. Spinach has also been shown to have the highest amounts of alpha-lipoic acid among plant sources [20]. Alpha-lipoic acid is considered as a micronutrient and a powerful antioxidant which has high antioxidant capacity and contributes to mitochondrial activity and has a positive effect on exercise performance [21]. In this regard, Rouhi et al. (2021), showed that consuming spinach supplements in healthy trained men after two half marathons increased total antioxidant capacity (TAC) and decreases MDA as an indicator of oxidative stress [22]. In another study, Gonzales et al. (2021), reported that a 7-day red spinach supplementation significantly reduced the time of performing a cycling time-trial task [23]. Accordingly, consuming spinach is deemed an effective nutritional strategy to counteract the detrimental effects of oxidative stress and improve athletic performance [24–26].

Another natural nutrient that has recently been gaining attention, particularly for its anti-inflammatory and immune system enhancing effects, is nutrition bio-shield superfood (NBS) [27–30]. The NBS is a new, organic, and healthy herbal supplement and contains macro and micro molecules, various vitamins including B1, B2, B3, B5, B6, B9, C, D, K, E, and the minerals such as magnesium, potassium, phosphorus, manganese, calcium, iron, copper, zinc, omega-3, omega-6, omega-9, and other substances [30, 31]. In one of the recent studies in this context, Azizi et al. (2022), indicated that the NBS powder could provide advantageous anti-inflammatory effects in patients COVID-19 [29]. In another study, Mohammadi et al. (2023), demonstrated that the NBS supplement is efficient in reducing stress and anxiety among women with depression and obesity [32]. Nevertheless, despite such promising effects in studies using NBS, there is a dearth of information regarding the effectiveness of this product in humans, which highlights the importance of conducting more studies to shed light on this interesting topic and bridge the current gaps in the literature.

For instance, in most of the previous studies investigating the effects of nutritional supplements on oxidative stress, aerobic exercise or resistance exercise has been used as the training protocol, nevertheless, many sporting activities involve short bouts of maximal or all-out effort ranging from a few seconds to less than one minute interspersed with periods of sub-maximal activities in between, also known as anaerobic activity [33]. In such activities, metabolic perturbations lead to a high accumulation of metabolic by-products and an increase in oxidative stress [34], which last from hours to a few days after the exercise [7, 8, 35, 36]. Unfortunately, despite the relevance of using nutritional supplements to cope with the repeated anaerobic-induced oxidative stress, this area of study has almost been overlooked. Moreover, to the best of our knowledge, no study so far has compared the efficacy of a well-established nutritional ergogenic aid like spinach plant with new promising supplements like NBS for oxidative stress caused by repeated all-out anaerobic activities.

Therefore, in the present study, we aimed to investigate the effects of a 7-day spinach or NBS superfood supplementation on IL-6, SOD, and MDA after performing repeated bouts of the Wingate test. We hypothesized that 7-day spinach or NBS superfood supplementation would decrease IL-6 and MDA, and increase SOD after performing repeated all-out (30-s Wingate test) activities.

Methods

Participants

Fifteen healthy and active men voluntarily participated in the study. The participants characteristics are presented in Table 1. The sample size was calculated using G*Power software (Version 3.1.9.2, Kiel, Germany) software as follows: test family = F tests; Statistical test = ANOVA: Repeated measures, within factors; α error probability = 0.05; power (1-β err prob) = 0.80; Effect size f = 0.35, number of groups = 1, number of measurements = 3, Correlation among repeated measures = 0.5, and Non-sphericity ε correction = 1. Accordingly, 15 participants were appropriate as the sample size for the present study. The inclusion criteria were (a) age range of 18 to 30 years, (b) Body Mass Index (BMI) 18.5 to 24.9 kg/m2, and (c) history of familiarity and performing anaerobic exercises. The exclusion criteria were (a) developing any type of cardiovascular, pulmonary, and metabolic disease, (b) history of seizures, epilepsy, or other types of neurological diseases, (c) smoking and alcohol use. Also, the participants who voluntarily wanted to withdraw from the study, faced with any acute diseases during the intervention phase of the study, and did not participate in any of the experimental session were excluded from the study and final analysis. This study was approved by the Ethics Committee of Razi University (Approval code: IR.RAZI.REC.1403.013; Date: 22.04.2023).

Study design and randomization

This study had a within-subject counter-balanced design with a control condition. And. The order of participants' exposure to 3 different conditions (spinach/NBS/control) was randomized by the Latin Squares method a publicly available website (www.randomization.com). To do so, first using the site www.random.org, a number between 1 and 15 was randomly allocated to each participant as an identification code. Then, the English letters A, B, and C was assigned to three intervention conditions and a Latin Square with three rows and three columns was created. Finally, participants 1 to 5 were placed in the sequence of the first row, participants 6 to 10 were placed in the sequence of the second row, and participants 11 to 15 were placed in the sequence of the third row.

Table 1 General characteristics of participants

Variables	Mean ± SD
Age (years)	22.1 ± 3
Body Mass (kg)	60.71 ± 2.7
Height (cm)	**176.89 ± 1.4**
BMI (kg/m^2)	**21.14 ± 1**
Body Fat (%)	**17.6 ± 3.1**
Fat Mass (kg)	**12.55 ± 4.9**
Resistance Training Experience (years)	**4.2 ± 1.8**

Experimental procedure

First, the participants participated in a familiarization session to be acquainted with whole stages of the research, performing the tests, and measuring the study variables. The anthropometric characteristics of the participants were measured (InBody, ZEUS 9.9, South Korea) in the same session. To avoid any abnormal reaction to spinach and NBS superfood[1] consumption, participants were given a dose of spinach and superfood. None of the participants reported abnormal reaction within 48 h after consuming spinach and NBS superfood. Then, the participants were randomly assigned to three different conditions including: (1) seven days of spinach supplementation; (2) seven days of NBS superfood supplementation; and (3) control condition. Seven days of wash-out were applied between the conditions. The duration of nutritional supplementation and the wash-out period were based on previous studies [37, 38]. The experimental procedure for the participants was as follow, the first blood sample was taken before being exposed to the experimental condition as the pre-test for the respective condition (spinach, NBS superfood, or control). Then, the participants were exposed to 7 days of either spinach or NBS superfood supplementation, or being under control condition. 24 h after the end of each condition, the participants came to the lab an performed 3 bouts of 30-s Wingate test with 4 min of recovery between each bout. 24 h after performing the Wingate test, the second blood sample were taken as the post-test for the respective condition. Then, the participants had 7 days of wash-out period and then, the same procedure was applied for the second and third condition. The amount of use of spinach or the dose of spinach for each participant was 70 g of fresh raw spinach packed per day before bedtime. The serving size was calculated using the USDA National Nutrient Database was standard reference [28, 39]. The NBS superfood supplement dose was 10 g per day before bedtime [28]. A 24-hour paper-based dietary recall was applied by a nutrition expert (through an interview with each participant) before the first experimental session and participants were instructed to follow the same diet 24 h before the next two experimental sessions (the sessions they performed the Wingate test). Moreover, to avoid any effects of circadian rhythm on the study variables, each participant came to the laboratory at the identical time of the day in a laboratory-controlled ambient condition (19–22 °C; 50–60% relative humidity) in all experimental sessions.

[1] 1 NBS organic company.

Blood sampling and sample measurement

Before receiving the supplement and starting each condition, the participants attended the laboratory and a blood sample of 6 ml of Cubital fossa was taken. It should be noted that all blood samples were taken in fasting state (at least 8 h of fasting) by laboratory experts of the hospital. Another sample was taken 24 h after the training session (three bouts of Wingate test). Blood plasmas were stored at -80 °C until further tests were performed. To separate the serum, the serum was placed at room temperature for 20 min to clot, then blood samples were centrifuged at 3000 rpm for 15 min and were used to measure blood factors using standard kit. The levels of IL6, MDA, SOD was measured by kits (96/48 Tests) ZellBio GmbH (Germany) CAT No، photometry with a spectrophotometer with a wavelength of 546 n.

Spinach and NBS superfood supplementation

The amount of use of spinach or the dose of spinach for each participant was 70 g of fresh raw spinach packed per day before bedtime. The serving size was calculated using the USDA National Nutrient Database was standard reference [28, 39]. The NBS superfood supplement dose was 10 g per day before bedtime [28]. NBS superfood supplement is known as an herbal supplement that has therapeutic properties [28–30, 32]. This herbal supplement is basically prepared from grains (mostly wheat), which have been enriched and improved the properties of this supplement. In terms of nutritional value, this organic supplement contains 25% protein, 50% carbohydrates, 15% fiber, 10% omega-3 and omega-6 and other vitamins. The daily dose of superfood for each participant was considered to be 10 g; According to reliable scientific sources, it is suggested that 4.5 g [28]. should be consumed daily for each non-athlete and healthy person, and this dose can be changed in different people with different physical characteristics and duration of consumption up to 20 g per day.

Repeated anaerobic performance (wingate test)

The participants performed three bouts of the 30-s cycling all-out Wingate test with 4 min of active recovery on a cycle ergometer (Ergomedic 894E, Monark Sports and Medical, Stockholm, Sweden). The bike settings (saddle height) were kept the same in all experimental sessions, according to each preference set in the familiarization session. A standard 5-minute warm-up was performed, consisting of pedaling with a 2% resistance of total body weight and performing 3 sprints of 5s at minutes 2, 3, and 4 with a 6.6% resistance of total body weight. After the warmup and before starting the test, a 3-min passive recovery was applied, and then, with the command “Go”, the participants started pedaling as fast as possible for 30s against a constant load equal to 7.5% of each participant’s body mass. After each bout of the Wingate test, the participants performed 4 min of active recovery on the bike with 30–40 W at 50 rpm. During the last 10 s of the recovery, the participants were informed to be ready for the next bout and the last 3 s of the recovery phase were counted down by the experimenter to inform the participants of the start point of the next bout of the Wingate test. Strong verbal encouragement was provided during each bout of the Wingate test. The Peak power (PP: highest power output achieved during the 30 s test) and mean power (MP: average power calculated for the complete test duration) were obtained in each bout. The fatigue index was subsequently calculated [FI = (Peak Power - Minimum Power) / (Peak Power × 100)] [40, 41].

Statistical methods

Data are presented as the mean ± standard deviation (M ± SD). The normal distribution of each data set was evaluated by the Shapiro-Wilk normality test. Two-way repeated measures ANOVA (3 × 2 factorial design; 3 stimulation conditions and 2-time points) was used to analyze IL-6, SOD, and MDA at pre- and post-test. Bonferroni post hoc test was used for the pairwise comparisons. In case of a violation in the assumption of sphericity, the Greenhouse-Geisser epsilon correction was applied. Partial eta squared (η^2_p) was used as a measure of the effect size for the ANOVAs and interpreted as small (0.01–0.059), medium (0.06 to 0.139), or large (≥ 0.14). The statistical analyses were performed using SPSS 27 (SPSS Inc., Chicago, IL, USA) and p<0.05 was adopted.

Results

The overall results of the study variables are presented in Table 2.

The results showed significant main effects of condition ($F_{(1.1,\ 16.6)} = 43.43$, $p < 0.001$, $\eta^2_p = 0.756$), time ($F_{(1.0,\ 14)} = 103.3$, $p < 0.001$, $\eta^2_p = 0.881$), and also

Table 2 Mean values of the study variables at pre and post intervention under 3 different conditions

	Experimental Conditions					
	NBS		Spinach		Control	
Variables	Pre	Post	Pre	Post	Pre	Post
IL-6$_{(pg/ml)}$	3.68 ± 1.1	11.22 ± 1.7	3.77 ± 1.2	5.86 ± 1.3	4.4 ± 1.41	18.03 ± 6.58
SOD$_{(U/mL)}$	26.75 ± 14.4	36.16 ± 10.9	28.46 ± 9.5	37.53 ± 12.0	26.83 ± 7.8	27.25 ± 7.24
MDA$_{(\mu M)}$	3.22 ± 1.6	2.3 ± 0.2	2.85 ± 0.6	2.24 ± 0.16	2.14 ± 0.5	2.68 ± 0.35

"condition × time" interaction *($F_{(1.1,\ 16.5)}=35.43$, $p < 0.001$, $\eta^2_p = 0.717$)* on the **IL-6**. Pairwise comparisons revealed that the **IL-6** was significantly lower at post-test in spinach and NBS superfood conditions compared to the control *($p < 0.001$;$p=0.003$, respectively).* The results also demonstrated that the **IL-6** was significantly lower at post-test in the spinach condition than in the NBS superfood condition *($p < 0.001$)*(Fig. 1).

There were significant main effects of condition *($F_{(2,\ 26)}=19.5$, $p < 0.001$, $\eta^2_p = 0.600$)*, time *($F_{(1,\ 13)}=23.1$, $p < 0.001$, $\eta^2_p = 0.640$)*, and also "condition × time" interaction *($F_{(1.2,\ 16.4)}=18.38$, $p < 0.001$, $\eta^2_p = 0.586$)* on the **SOD**. Pairwise comparisons indicated that at post-test, the SOD was significantly higher in the spinach *($p < 0.001$)* and NBS superfood *($p < 0.001$)* conditions compared to the control condition. No significant difference was observed between the spinach and NBS superfood conditions; (Fig. 2).

MDA

Our results demonstrated a significant "condition × time" interaction effect *($F_{(1.1,\ 14.9)}=7.04$, $p=0.015$, $\eta^2_p = 0.351$)* on the **MDA**, while no significant main effects of condition *($F_{(1.3,\ 16.9)}=0.96$, $p=0.39$, $\eta^2_p = 0.069$)* and time *($F_{(1,\ 13)}=4.61$, $p=0.051$, $\eta^2_p = 0.262$)* were seen on the **MDA**. Pairwise comparisons revealed that the **MDA** was significantly lower in the spinach *($p < 0.001$)* and NBS superfood ($p=0.017$) conditions compared to the control condition in post-test; (Fig. 3).

Discussion

The results of the present study showed that under control condition, 24 h after performing repeated all-out anaerobic activity, the IL-6 and MDA was higher compared to the baseline which shows that such activities might induce oxidative stress. This in line with the results of the previous studies reporting an increment in markers of the oxidative stress and inflammatory response following demanding anaerobic activities [42, 43]. Interestingly, the results indicated that under spinach and NBS supplementation, the IL-6 and MDA were significantly lower 24 h after performing the exercise protocol compared to the control condition.

It has been demonstrated that spinach could alleviate oxidative stress by modulating antioxidant metabolism, leading to a protection against detrimental effects of oxidative stress [44]. In this regard, Bohlooli et al. (2014), also reported that 14 days of supplementation with spinach was able to attenuate the concentration of oxidative stress and muscle damage caused by performing a half-marathon in healthy trained young men [26]. In a study by Arru et al. (2021), they reported that there is a mixture of natural antioxidants in spinach leaves which contain aromatic polyphenols, including the phenolic acids and

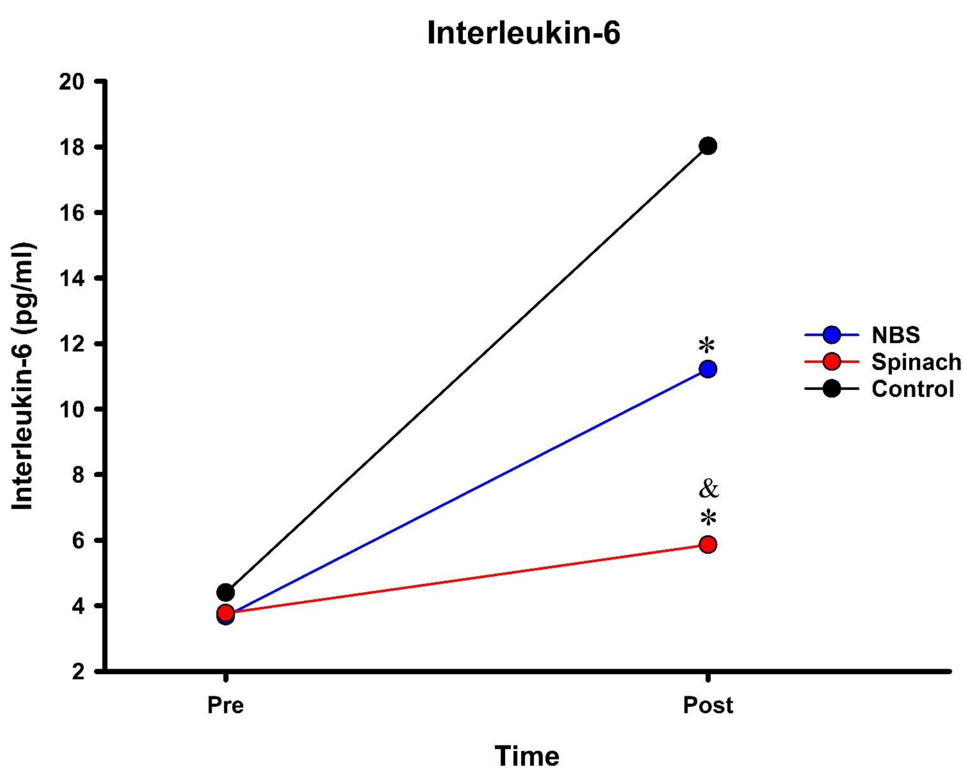

Fig. 1 Interleukin-6 levels changes in pre-test and post-test (24 h after Wingate test) in three different conditions *= significant difference compared to the control condition; &= Significant difference compared to the NBS condition

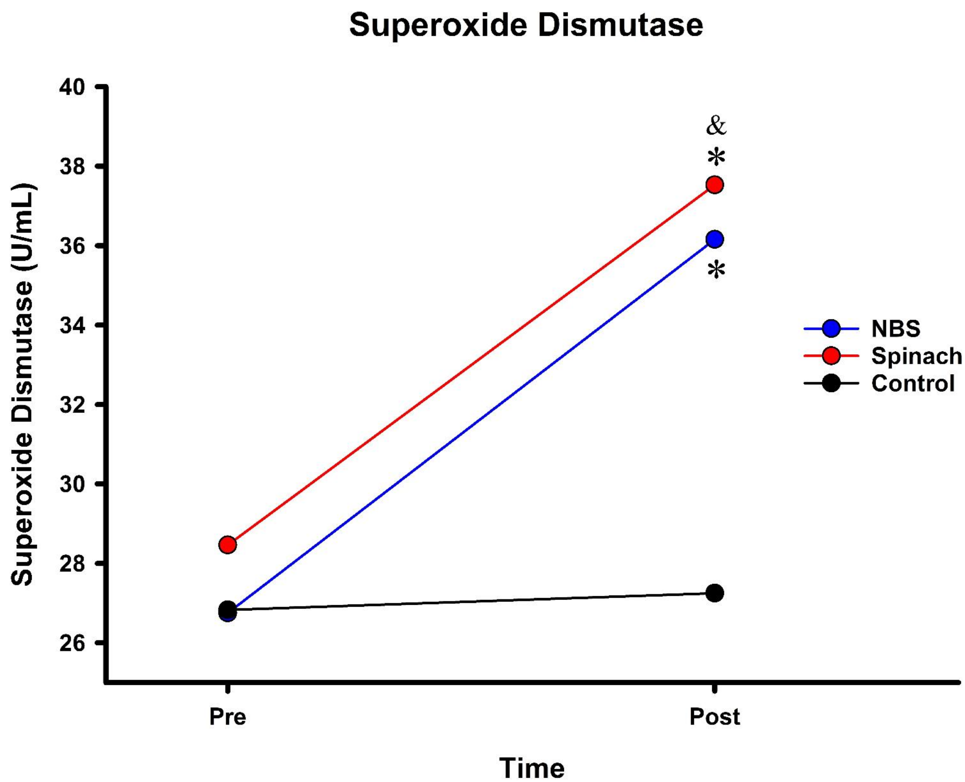

Fig. 2 Superoxide dismutase levels changes in pre-test and post-test (24 h after Wingate test) in three different conditions *= significant difference in comparison with the control condition; &= significant difference in comparison with the NBS condition

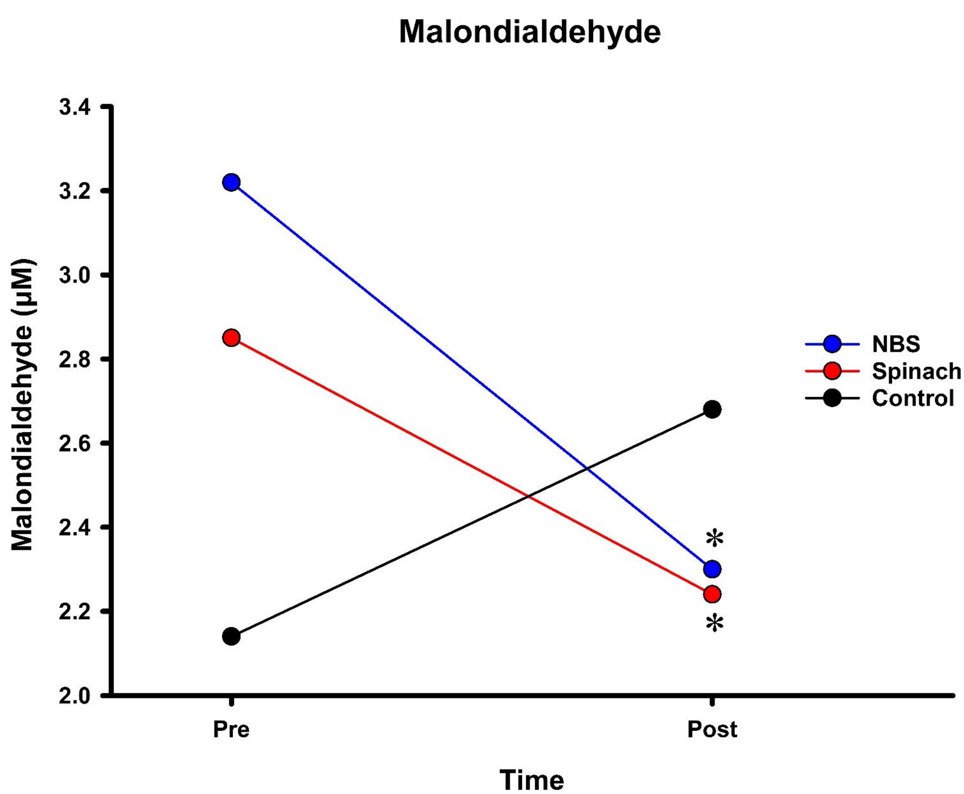

Fig. 3 Malondialdehyde levels changes in pre-test and post-test (24 h after Wingate test) in three different conditions *= significant difference in comparison with the control condition

the derivatives of the glucuronic acid. The natural antioxidants are effective in coping with free radicals, leading to an antiproliferative and anti-inflammatory potentials [45]. This could somehow explain the positive effects of a 7-day spinach supplementation on markers of oxidative stress such as IL-6 and MDA 24 h after performing a repeated all-out anaerobic task in the present study.

Contrary to spinach supplementation, there is dearth of information on the efficacy of NBS for sports performance and coping with exercise-induced oxidative stress. However, in one of few studies using NBS supplementation reduced the markers of oxidative stress and inflammation such as IL-6 and TNF-α [28]. Likewise, Azizi Jalilian et al. (2022), indicated that NBS supplementation brought about a reduction in IL-2, IL-6, and TNF-α in patients with COVID-19 [29]. Based on such studies, it seems that the NBS supplementation not only can be effective in coping with oxidative stress and inflammation in clinical condition but also can be considered effective in alleviating exercise-induced oxidative stress in healthy population. Unfortunately, no specific mechanisms have been suggested in the previous studies on how NBS supplementation could address oxidative stress and inflammation.

Our results also revealed that 24 h after performing the exercise protocol, the serum level of the SOD was significantly higher in spinach and NBS conditions compared to the control condition. Normally, an exercise-induced ROS production is followed by an augmentation in the activity of antioxidant enzymes such as SOD in order to neutralize the harmful effects of ROS [46]. It seems that 7 days of supplementation with either spinach or NBS was able to increase SOD as an antioxidant agent [30, 44]. SOD is an enzyme with a well-known therapeutic activity against ROS by breaking down potentially harmful oxygen molecules in cells [16]. Therefore, since SOD prevents the accumulation of ROS, it could be inferred that it is probably one of the potential mechanisms by which the spinach and NBS supplementation reduced the levels of IL-6 and MDA 24 h after performing the exercise protocol in the present study.

Although all the necessary details were taken into account to ensure optimal control of the study procedure, the results of the present study must be interpreted with caution, as they are not free from the effects of limiting factors. Only three markers related to the oxidative stress were measured at pre-test and 24 h after performing the exercise protocol in the present study, which limits the comprehensive interpretations of the results. In addition, despite we instructed the participants regarding their diet during the experiment to ensure the homogeneity of three conditions, we were not able to have a complete control over the diet, which must be taken into consideration when interpreting the results of the present study. On the other hand, the strength of the present study includes the fact that we investigated possible ergogenic aids of nutritional supplementation to cope with the oxidative stress in response to repeated anaerobic activities, which has been less investigated in the previous studies. Furthermore, for the first time to the best of our knowledge, we compared the possible effects of spinach, as a previously well-established nutritional aid, with the NBS, as a recently developed nutritional supplement with promising anti-inflammatory and immune enhancing effects, which could be considered a novel contribution of the present study.

Conclusion

The present study suggests that consuming spinach and NBS could be deemed a protentional nutritional intervention to deal with the increase in the markers of oxidative stress caused by performing repeated anaerobic all-out activities. Moreover, when comparing the efficacy of these two nutritional strategies, spinach supplementation is more effective in reducing markers of the oxidative stress after performing a demanding repeated anaerobic task. Future studies focusing on other markers of oxidative stress, more time points related to changes in such markers after anaerobic tasks, and different time and dosage of supplementation are warranted to shed more light on this field of study.

Abbreviations

SOD	Superoxide dismutase
MDA	Malondialdehyde
IL-6	Interleukin 6
NBS	Nutrition bio-shield superfood
ROS	Reactive oxygen species

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Author contributions

MMSH, MA, EA, conceptualized and designed the study. MMSH, MA, EA conducted the experiments. MMSH, MA participated in the formal analysis. MA wrote the original draft of the manuscript. MMSH, MA, EA, reviewed and edited the manuscript All authors approved the final version of the manuscript. MA supervised the project.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Razi University (Approval code: IR.RAZI.REC.1403.013; Date: 22.04.2023). All the experimental procedures were conducted following the declaration of Helsinki. Informed consent was obtained from all subjects before participation in the study.

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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