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Antioxidant supplementation boosts the advantages of CrossFit workouts on oxidative and muscle damage markers in obese males

Morteza Nemati[1], Neda Bozorgtabar[2], Maha Hoteit[3,4], Zahra Sadek[4,5], Abdullah Almaqhawi[6], Ali Rashidy-Pour[7], Nematollah Nemati[8], Mohammad Rashidi[9], Niloofar Karimi[10], Mitra Khadamosharieh[11], Reza Bagheri[12], Ayoub Saeidi[13*], Maisa Hamed Al Kiyumi[14], Katie M. Heinrich[15] and Hassane Zouhal[16,17]

Abstract

Supplementing with antioxidants may be one of the most efficient means of minimizing oxidative stress during workouts in obese individuals. The aim of this study is to identify the results after twelve weeks of CrossFit workouts combined with Spinach thylakoid extract on the levels of insulin resistance (insulin and Homeostatic Model Assessment of Insulin Resistance [HOMA-IR]), fasting blood sugar (FBS), malondialdehyde (MDA), creatine kinase (CK), lactate dehydrogenase (LDH), total antioxidant capacity (TAC), and superoxide dismutase (SOD), glutathione peroxidase (GPx) in obese males. Sixty-eight males with an average age of 27 ± 8 yrs and a BMI of 32.6 ± 2.6 $kg.m^{-2}$ were randomly split into four groups each consisting of seventeen individuals. : control group (CG), supplement group (SG), training group (TG), and training + supplement group (TSG). After initial assessments, the two training groups (TG and TSG) started on a 12 weeks of the CrossFit workouts program involving three sessions per week each lasting up to 60 min. Participants in supplement groups ingested 30 min before lunch, 5 gof Spinach thylakoid extract per day or one sachet of raw corn starch in the control group. Baseline and post-intervention measurements were performed 48 h pre- and post-last session, respectively. The findings revealed noteworthy relationships between the exercise groups and timefor TAC, SOD, GPx, MDA, CK, and LDH ($p < 0.001$, ES: 0.88, 0.88, 0.8, 0.4, 0.7, and 0.7, respectively). In addition, there were statistically significant differences among study groups after attending the intervention program in TAC (ES: 0.88), SOD (ES: 0.92), GPX (ES: 0.85), MDA (ES: 0.5), CK (ES: 0.7) and LDH (ES: 0.8). The effect sizes of insulin (0.77), glucose (0.21), and HOMA-IR (0.44) varied significantly ($p < 0.05$) among the groups. The results demonstrated that CrossFit workouts for 12 weeks combined with Spinach thylakoid extract in men with obesity may prevent oxidative damage caused by obesity and CrossFit workouts.

Keywords CrossFit workouts, Oxidative stress, Muscle damage, Obese male

*Correspondence:
Ayoub Saeidi
a.saeidi@uok.ac.ir

Full list of author information is available at the end of the article

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Introduction

Obesity is a chronic disease and its prevalence has grown in countries that are both developed and developing. Obesity is a significant risk factor for many of the most prevalent diseases in the world, according to numerous research, including diabetes, cardiovascular disease, hypertension, metabolic disorders, and different types of cancers [1]. Also, the available evidence suggests that elevated oxidative stress is linked to obesity,, decreased human body antioxidant capacity, and lipid peroxidation [2]. Elevated levels of oxidative stress are a significant contributor to the metabolic syndrome related to obesity and could have a significant impact on the pathogenesis of various diseases including type 2 diabetes [3]. On the other hand, some studies suggest that regular exercise can be an effective means of preventing obesity and associated diseases [3]; However, vigorous exercise can cause free radicals to be produced, which can lead to oxidative stress and consequent cellular damage [4–6]. As a result, following physical activity, the body's ability to protect itself against oxygen free radicals by producing antioxidants is disrupted, a condition known as oxidative stress [7]. During the lipid peroxidation process, free radicals attack cellular components, especially unsaturated fatty acids in the cell membrane, producing more free radicals. In this way, broken-down fatty acids, hydrocarbon gases, and aldehydes are formed, causing muscle damage and inflammation [8]. In most studies, researchers use indicators like aldehydes, particularly malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity (TAC), and glutathione peroxidase (GPx) to measure oxidative stress [8–12]. To deal with the oxidative stress produced, the cell is well equipped with an enzymatic antioxidant defense system including SOD and GPx, and cellular CAT, which are the cell's first defense barrier against a variety of activated oxygen radicals [13]. There are reports of increased TAC in marathon runners, but this increase is insufficient against muscle damage and lipid peroxidation stimulated by exercise [13, 14]. It has also been shown that fat accumulation and body mass index (BMI) is positively correlated with oxidative stress indices. However, the effects of obesity and overweightness are reduced following participation in sports activities and the use of nutritional interventions, where the intensity and duration of exercise training and the nutritional strategies play a role in the process of reactive oxygen species (ROS) production [15]. Recent research has shown that, in addition to their negative consequences, the production of ROS has a number of beneficial impacts on sports performance, as well as a role in cell functions and response to regular workouts [16–18].

In recent years, due to the detrimental consequences of synthetic medications, utilizing medicinal and natural plants with antioxidant properties has captured the interest of the majority of sports coaches and researchers to protect against exercise-induced oxidative stress [19]. Many beneficial components found in spinach (Spinacia oleracea L.) include lutein, lycopene, omega-6 fatty acids, flavonoids, phenolic compounds, and β-carotene [20, 21]. It is hypothesized that these phytochemicals are responsible for spinach's hypoglycemic, hypolipidemic, anti-obesity, antioxidant, and anti-inflammatory qualities [22]. It is also a longtime source of flavonoid and p-coumaric acid all of which have antioxidant properties [23]. Therefore, spinach can protect against the deleterious effect of ROS [24]. Numerous research conducted on various animal models have demonstrated the antioxidant effects of spinach, including its impact on hyperlipidemia [25]. On the other hand, CrossFit workouts, an almost new method of physical activity, aims to train muscles through coordinated, multiplane movement patterns. This approach involves engaging multiple joints, dynamic tasks, and frequent adjustments in the base of support, all designed to enhance overall function [26, 27]. CrossFit workouts is shown to improve oxygen capacity, insulin resistance, and muscular strength [28], as well as the MetS z-score [29]. In healthy populations, CrossFit workouts has also improved cardiorespiratory fitness [30], muscular strength and power [31], and waist circumference and agility [32]. Jogging, cycling, and weight liftingand other activities are all part of the popular multimodal high-intensity training program known as CrossFit [33, 34]. The best training models are excuted in an intense circuit style with a small rest interval or no rest at all to enhance aerobic and anaerobic fitnessand body building [33, 34]. Due to the positive impact that medicinal plants can have on antioxidants and the extra stress caused by intense exercise, we conducted this study to test the hupothesis if adding spinach to the diet could help reduce oxidative stress from CrossFit workouts in obese men. Prior research has looked at the impacts of intense interval training on muscleinjury, oxidative stress, and inflammatory markers [35–37], Not many research have looked at the impact of of CrossFit workouts along with the use of antioxidant supplements on these indices [38, 39].

To our knowledge, there is no information as to how the supplementation of spinach combined with CrossFit workouts affects oxidative stress markers. Also, the question arises whether the adaptation resulting from 12 weeks of CrossFit workouts, without taking antioxidant supplements, can only affect the antioxidant indices. Therefore, the relationship between exercise, oxidative stress, and supplements is still complicated. Taken together, the present study aimed to evaluate the results after twelve weeks of CrossFit workouts with Spinach thylakoid extract on lipid profile, insulin resistance, and

select oxidative and muscle damage markersin obese males.

Materials and methods

Following calls to public locations like fitness centers, clinics, hospitals, and social media, 100 males volunteered for the present study, while 32 of them were not eligible to enter the study. After reviewing, 68 males were finally selected for the present study with an average age of 27±8 yrs, a height of 168.4±2.6 cm, a body massof 95.7±3.8 kg and a BMI of 32.6±2.6 kg.m^{-2}. Inclusion criteria encompassed a BMI exceeding 30 kg.m-2, a lack of regular physical activity in the preceding six months, no history of supplement and drug use in the past month, no documented history of endocrine, metabolic, or cardiovascular disorders, abstention from alcohol consumption and no history of smoking. Participants with arthropathy, functional impairements, and those currently using prescribed pharmaceuticals and dietary supplements that might influence the metabolism of muscle and adipose tissue were excluded from the study. Every participant performed a physical assessment given by an exercise specialist on the first visit. They also completed the Physical Activity Readiness Questionnaire (PAR-Q) and submitted a written consent form [40]. Every process followed the most recent edition of the Helsinki Declaration. The Iranian Registry of Clinical Trials (IRCTID: IRCT20151228025732N77) and the Research and Ethics Committee of the Islamic Azad University, Damghan branch (Ethics code: IR.IAU.DAMGHAN.REC.1401.034) approved the research methodology [41].

Experimental design

Following the evaluation of anthropometric and body composition metrics, participants were randomly assigned to one of four groups, each comprising seventeen individuals: control group (CG), supplement group (SG), training group (TG), and training+supplement group (TSG). Participants in the control group were instructed to maintain their regular daily activities until the conclusion of the study. Baseline assessments were conducted 48 h before the commencement of the training protocols, and post-tests were carried out across all groups 48 h after the final session. To ensure dietary consistency, participants in the training protocols adhered to the same diet one week before the baseline assessments and just prior to the final measurements. A familiarization session, elucidating all study procedures, took place one week before the initiation of training sessions. In the third session, where body composition variables and VO2peak were measured, each group received instructions on executing the training procedures. The two groups initiated the 12-week exercise training program, consisting of three sessions per week, following the baseline measurements.

Body composition and cardio-respiratory fitness assessments

To calculate BMI, body mass and height were measured using a calibrated scale and stadiometer (Seca, Germany). A bio-impedance analyzer (Medigate Company Inc., Dan-dong Gunpo, Korea) was used to measure the amount of fat mass (FM) and fat-free mass (FFM). An electronic sphygmomanometer (Kenz BPM AM 300P CE, Japan) was used to measure blood pressure, and a Polar V800 heart monitor (Finland) was used to record heart rate. A calibrated gas analyzer (Metalyzer 3B analyzer, Cortex: biophysics, GMbH, Germany) was used to do gas analysis before to each test.

VO2peak was assessed, following the standards set by the American College of Sports Medicine (ACSM), using a modified Bruce protocol on a treadmill (H/P/Cosmos, Pulsar med 3p- Sports & Medical, Nussdorf-Traunstein, Germany) consistent with previous studies involving overweight and obese populations [42, 43]. Physiological assessment of VO2peak was based on participants indicating their maximum effort and reaching exhaustion, as measured by the Borg scale, or if the supervisor observed severe dyspnea with a plateau in VO2 and respiratory exchange ratio (RER)≥1.10, following the guidelines for cardiopulmonary exercise testing (CPET) established by ACSM and the American Heart Association [44, 45].

Preparation of spinach thylakoids and placebo

Thylakoid membranes were prepared according to published protocols using fresh baby spinach leaves (Spinacia oleracea) from Tabriz, in the East Azerbaijan Province of Iran [46–48]. The thylakoid supplement used in this study was created on an experimental dimension in the Islamic Azad University Laboratory utilizing the approach described by Tabrizi et al. (2020) [49]. The eligible participants were designated to obtain 30 min before lunch for 12 weeks, 5 g of Spinach thylakoid extractSpinach thylakoid extract per day or one sachet of raw corn starch in the control group.

Training protocols

The CrossFit exercise plan involved 36 one-hour sessions led by a Level 1 CrossFit-certified trainer. The initial two sessions provided an overview of standard CrossFit motions without actual training. Starting from the third day, each session included 10–15 min of stretching and warm-up, 10–20 min of exercises, and 5–30 min for the Workout of the Day (WOD) performed at a high intensity as aerobic, body weight and weightlifting, tailored to each person's fitness level.During each CrossFit session, participants received personalized instructions outlining

specific weights, movements, completion times, rounds, repetitions, WOD structure, average times per session, and weekly average times [51]. Any adjustments needed from the planned workout were carefully calculated, documented, and recorded.

Blood markers

Every test was conducted between 8 and 10 am under typical operating conditions. The right arm was used to draw blood samples following a 12-hour fast, There was a 72-hour period both before the first workout and after the last workout.Samples of bloods were put into tubes, spun at 3000 rpm for 10 min, and then kept at -70 °C. Serum glucose levels were measured using a colorimetric enzymatic kit (Parsazmun, Tehran, Iran) with a sensitivity of 5 mg/dl. Insulin resistance was assessed using the homeostasis model evaluation (HOMA) formula: HOMA-IR = 22.5 μmol/(fasting plasma insulin × fasting plasma glucose) [52]. The Sandwich-ELISA method was used to measure the levels of LDH and CK in plasma; the assay ranges were (0.78-50 ng/ml) for LDH and (0.156 – 10 ng/ml) for CK.

The ELISA kit was used to measure:

- Serum SOD (MyBioSource, San Diego, CA, USA) with a sensitivity of 1 ng/mL. The intra-assay coefficient of variation was less than 8%, and the inter-assay coefficient of variation was less than 12%.
- TAC (MyBioSource) with a sensitivity of 0.14 U/mL. The intra-assay coefficient of variation was less than 8%, and the inter-assay coefficient of variation was less than 10%.
- GPx (ZellBio, Lonsee, Germany)with a sensitivity of 5 U/mL. The intra-assay coefficient of variation was 3.5%, and the inter-assay coefficient of variation was 4.7%.
- MDA (BioCore, Germany) with a sensitivity of 6.4 μmol/L.

Using enzymatic techniques, Serum total cholesterol (TC) and triglyceride (TG) were determined (CHOD-PAP). A photometric approach was used to measure the levels of high-density cholesterol (HDL-C) and low-density cholesterol (LDL-C) using a Pars Testee's Quantitative Detection kit from Tehran, Iran. For LDL-C, the method showed a sensitivity of 1 mg/dl and a coefficient of 1.2%, whereas for HDL-C, the results were 1.8% and 1.2%, respectively. Insulin levels were measured using an ELISA kit from Demeditec, Germany, which showed a sensitivity of 1 ng/ml. The measurements' within-coefficients of variance varied from 5.1 to 8.4%.

Statistical analysis

The sample size justification was based on prior research on interval exercise, utilizing G-power 3.1.9.2. The study aimed to investigate markers in a population with no previous CrossFit intervention study. Prior studies showed a significant increase in SOD with an effect size (ES) of 0.45. Using an F test, repeated measures, and within-between interaction ANOVA, a total sample size of at least 28 participants ($n=7$ per group) was needed for sufficient power ($\alpha=0.05$, power of 0.95). However, to account for potential impacts of COVID-19 on training and adherence to supplementation, and the absence of CrossFit studies on the measured adipokines, the sample size was increased to 17 participants per group to uphold the statistical power of the study.

To characterize all of the data, the study used descriptive statistics (means ± standard deviation). The Shapiro-Wilk test was used to determine whether the data were normal. A two-way ANOVA repeated measures test was used to identify the Group × Time interaction. One-way ANOVA and Fisher LSD posthoc tests were used to assess the baseline data for each of the four groups. When significant differences were found by ANOVA, pairwise comparisons were performed. To find statistically significant differences with a 95% confidence interval and a power value of at least 80%, sample size calculations were carried out. Hopkins et al. (2009) reported effect sizes (ES) as partial eta-squared and classified them as trivial (<0.2), small (0.2–0.6), moderate (0.6–1.2), large (1.2-2.0), and very large (2.0–4.0). Less than 0.05 was regarded as a statistically significant p-value. The SPSS program (version 24, Armonk, NY, USA) was used to analyze the data.

Results

Antioxidant and muscle damage markers

The values in pre and post-tests are shown in Fig. 1. Baseline levels of TAC, SOD, GPx, MDA, CK, and LDH were the same for all groups ($p>0.05$). The workout groups' significant interactions were found by the repeated-measures ANOVA test (i.e., TG, TSG) and time for TAC, SOD, GPx, MDA, CK, and LDH ($p<0.001$, ES: 0.88, 0.88, 0.8, 0.4, 0.7 and 0.7, respectively). Intra-group analyses demonstrated variations in post-test values for TAC (ES: 0.91), SOD (ES: 0.8), GPx (ES: 0.9), MDA (ES: 0.7), CK (ES: 0.8), and LDH (ES: 0.9) across all groups compared to their respective pretest values ($p<0.05$), with no observed changes in the control group (CG) ($p>0.05$).In addition, there were statistically significant differences among study groups after attending the intervention program in TAC (ES: 0.88), SOD (ES: 0.92), GPx (ES: 0.85), MDA (ES: 0.5), CK (ES: 0.7) and LDH (ES: 0.8) ($p<0.05$).

All main outcomes across the research groups showed statistically significant pairwise comparisons ($p<0.05$)

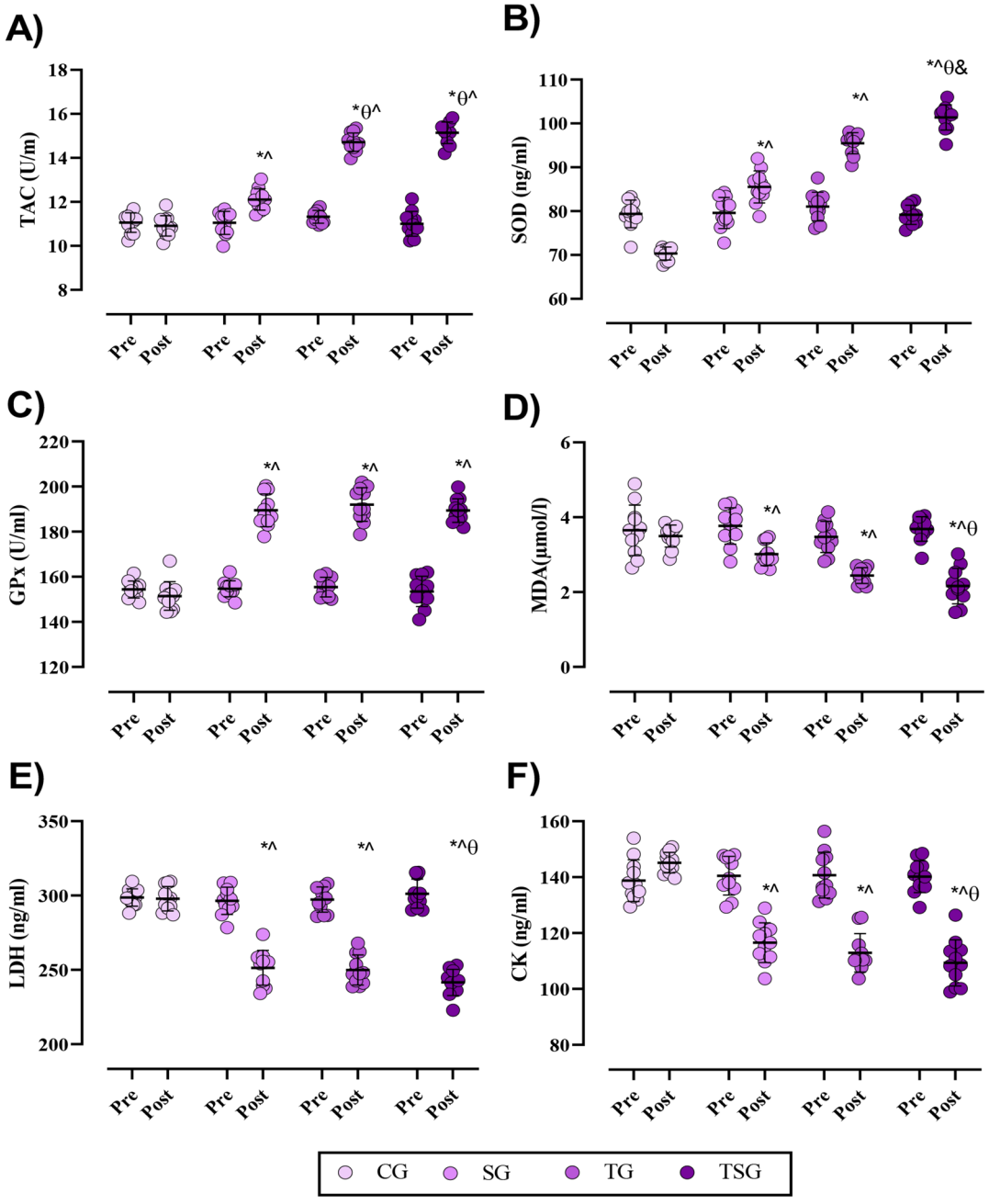

Fig. 1 The changes of antioxidants and muscle damage markers throughout the study. (**A**) Total antioxidant capacity (TAC); (**B**) Superoxide dismutase (SOD); (**C**) glutathione peroxidase (GPx); (**D**) Malondialdehyde (MDA); (**E**) Lactate dehydrogenase (LDH); (**F**) Creatine kinase (CK). $n=11$ per group, error bars represent SD. $ SG vs. TG; ^ all with CG ; & TSG vs. TG ; θ TSG vs. SG ; * vs. pre test

according to the post hoc multiple comparisons analysis, with the exception of the TSG and TG pair in GPX ($p=0.35$), MDA ($p=0.06$), CK ($p=0.2$), and LDH ($p=0.053$).Additionally, the pair of SG and TG in GPx ($p=0.4$), CK ($p=0.2$), and LDH ($p=0.7$) did not show statistically significant differences. Finally, the level of GPx ($p=0.4$) did not show a statistically significant difference between the pairwise comparisons of the SG and TSG ($p=0.9$). Levels of TAC, SOD, and GPx showed significant increases in the three intervention protocols (TG, SG, and TSG) compared to the CG ($p<0.05$). The increase was most pronounced in the TSG, followed by the TG and SG protocols ($p<0.05$). Conversely, levels of MDA, CK, and LDH were significantly decreased in the TG, SG, and TSG compared to the CG ($p<0.05$). The greatest reduction was observed in the TSG, followed by the TG and SG protocols ($p<0.05$).

Insulin, glucose, and HOMA-IR

Significant differences of the effect size in insulin (0.77), glucose (0.21), and HOMA-IR (0.44) between the groups are seen in Table 1 ($p<0.05$). According to within-group analyses, there were no significant changes in the CG ($p>0.05$), while the post-test levels of these variables in the TG, TSG, and SG were significantly lower than their respective pretest values ($p<0.05$). Additionally, for insulin, glucose, and HOMA-IR, the group × time interactions were statistically significant ($p<0.05$). The insulin, glucose, and HOMA-IR post hoc multiple comparisons analysis showed significant differences ($p<0.05$) in all pairwise comparisons between the study groups and the control group as well as between all other pairs, with the exception of the TG and TSG pair, which did not show any significant difference in HOMA-IR ($p=0.9$) or glucose levels ($p=0.33$). Moreover, insulin, glucose, and HOMA-IR results for TSG participants were considerably lower ($p<0.05$) than for SG participants.

Anthropometry, body composition, and cardio-respiratory fitness variables

In relation to anthropometry, body composition, and cardiorespiratory fitness characteristics (Table 1), there were no statistically significant variations found in the groups' BMI ($p=0.11$, ES: 0.14) or VO2 peak ($p=0.027$, ES: 0.09). Other variables, such as body mass with effect size (0.37), FFM (0.23), fat mass (0.42), HDL-C (0.37), LDL-C (0.34), TC (0.46), and TGs (0.66) ($p<0.05$), showed significant differences.The CG did not change in the within-groups comparison of the variables related to anthropometry, body composition, and cardiorespiratory fitness ($p>0.05$).

On the other hand, SG, TG, and TSG post-test values differed significantly ($p<0.05$) from pretest values. Additionally, all of the mentioned variables had statistically significant group and time interactions ($p<0.05$, ES range: 0.43–0.96).With the exception of BMI ($p=0.62$), VO2 peak ($p=0.53$), TGs ($p=0.78$), and HDL ($p=0.22$) in the SG, post hoc multiple comparisons of anthropometry, body composition, and cardiorespiratory fitness measures showed significant changes between the intervention groups and the control group ($p<0.05$). With the exception of body mass ($p=0.06$), BMI ($p=0.87$), and FFM ($p=0.78$), multiple pairwise comparisons between the SG and TG were statistically significant ($p<0.05$). No significant differences were found in body mass ($p=0.57$), VO2 peak ($p=0.78$), fat mass ($p=0.64$), HDL ($p=0.78$), LDL ($p=0.68$), TC ($p=0.31$), or TGs ($p=0.1$) when TG and TSG were compared. With the exception of FFM ($p=0.14$), all anthropometry, body composition, and cardiorespiratory fitness indicators showed a significant difference ($p<0.05$) between TSG and SG subjects.

Correlations

Initially, a correlation matrix (Fig. 2A) was created to investigate possible relationships between exercise-induced changes in fat mass (Δ FM) and changes in oxidative stress indicators (Δ marker, independent of groups). While MDA (Fig. 2E) showed a slight positive correlation with Δ FM, TAC (Fig. 2B), SOD (Fig. 2C), and GPx (Fig. 2D) showed slightly adverse correlations with Δ FM. Using the additional sum-of-squares F test, linear regression analyses were performed for each unique Δ (oxidative stress marker) as a function of Δ FM to determine whether the pooled data could be regarded as a single model. Without focusing on any one group, all the data showed a strong correlation with changes in FM ($p<0.001$).

Discussion

In the present study, we investigated the individual and concomitant effects of 12 weeks of CrossFit workouts and Spinach thylakoid extract on selected antioxidant and muscle damage markers, glucose homeostasis parameters and lipid profile, and body composition variables in obese males.

Our results show that 12 weeks of CrossFit workouts and Spinach thylakoid extract have caused a significant increase in post-test values of TAC, SOD, and GPx while decreasing MDA. Also, we noted significant decreases in muscle damage markers (CK and LDH), glucose homeostasis parameters (insulin, glucose, and HOMA-IR), blood lipids (TC and LDL), and factors related to body composition (body mass, FM, and FFM), in each experimental group when compared to pre-test levels (TG, SG, TSG). The improvements in the TSG were better than the SG and TG. Obesity is linked to increased mortality rates and is a risk factor for a number of health issues, including hyperlipidemia, hypertension, cardiovascular disease, cancers, type 2 diabetes mellitus, and insulin resistance [42, 53, 54]. However, other study supported our findings, which suggested that dietary modifications and exercise training could gradually reduce obesity-related lipotoxicity, increased oxidative stress, and release of pro-inflammatory cytokines [42, 53, 54]. Our data are consistent with a previous study that reported 8 weeks of endurance, resistance, and concurrent training significantly increased SOD, GPx, and TAC and decreased the level of MDA in untrained males [55]. Similarly, Bogdanis et al. reported that 3 weeks of high-intensity interval training (HIIT) induced a marked elevation of GPx and TAC in healthy humans [56]. Physical exercise increases the production of ROS and lipid peroxidation in skeletal muscle [55, 56]. However, as demonstrated by the current study, regular exercise can strenghthen the body's defences against oxidative stress and lower lipid peroxidation and oxidative stress, particularly in the case of overweight or

Table 1 Mean ± SD and effect size of anthropometry, body composition, and cardio-respiratory fitness variables

Variables	Group	Pre-training Mean (SD)	Post-training Mean (SD)	P values (η2)		
				T	G × T	G
Body mass (kg)	CG	94.33 (1.82)	93.55 (2.43)	$p < 0.001$ (0.63)	$p < 0.001$ (0.44)	$p < 0.001$ (0.37)
	SG	93.28 (2.61)	91.13 (2.12)			
	TG	92.78 (1.89)	89.19 (2.37)			
	TSG	94.13 (1.90)	87.25 (2.30)			
BMI (kg/m^2)	CG	33.08 (1.34)	32.87 (1.44)	$p < 0.001$ (0.63)	$p < 0.001$ (0.45)	0.11 (0.14)
	SG	32.66 (1.37)	31.93 (0.93)			
	TG	33.22 (1.07)	31.85 (1.19)			
	TSG	33.05 (0.75)	30.68 (0.95)			
VO_2peak ($mL \cdot kg^{-1} \cdot min^{-1}$)	CG	27.27 (2.41)	26.90 (2.25)	$p < 0.001$ (0.62)	$p < 0.001$ (0.62)	0.27 (0.09)
	SG	27.09 (2.84)	27.54 (2.91(			
	TG	27.36 (2.37)	29.90 (1.92(			
	TSG	27.00 (2.04)	30.18 (2.22)			
FFM (Kg)	CG	27.63 (1.20)	26.54 (2.25)	$p < 0.001$ (0.46)	$p < 0.001$ (0.43)	0.01 (0.23)
	SG	27.09 (1.81)	29.36 (0.92)			
	TG	26.72 (1.27)	29.54 (1.50)			
	TSG	27.18 (1.77)	30.36 (1.20)			
FAT (%)	CG	30.09 (1.51)	30.83 (2.05)	$p < 0.001$ (0.54)	$p < 0.001$ (0.46)	$p < 0.001$ (0.42)
	SG	30.10 (1.59)	28.08 (0.79)			
	TG	30.36 (1.50)	26.89 (0.95)			
	TSG	31.13 (1.35)	26.62 (1.21)			
HDL (mg/dl)	CG	39.34 (1.22)	38.43 (1.30)	$p < 0.001$ (0.72)	$p < 0.001$ (0.73)	$p < 0.001$ (0.037)
	SG	38.88 (1.23)	39.74 (3.99)			
	TG	38.66 (1.67)	44.51 (1.34)			
	TSG	38.56 (1.41)	44.80 (2.12)			
LDL (mg/dl)	CG	125.22 (4.47)	125.02 (4.70)	$p < 0.001$ (0.91)	$p < 0.001$ (0.86)	0.001 (0.34)
	SG	125.56 (5.42)	121.41 (5.24)			
	TG	126.75 (4.38)	111.20 (2.92)			
	TSG	127.14 (3.64)	110.50 (2.52)			
TC (mg/dl)	CG	226.70 (5.27)	226.81 (5.26)	$p < 0.001$ (0.97)	$p < 0.001$ (0.96)	$p < 0.001$ (0.46)
	SG	227.44 (5.48)	222.09 (5.19)			
	TG	227.81 (5.29)	207.44 (4.95)			
	TSG	227.38 (5.49)	205.23 (4.77)			
TGs (mg/dl)	CG	242.10 (4.39)	242.81 (3.85)	$p < 0.001$ (0.90)	$p < 0.001$ (0.89)	$p < 0.001$ (0.62)
	SG	245.83 (5.93)	242.05 (5.40)			
	TG	244.58 (7.48)	217.39 (9.88)			
	TSG	242.92 (5.96)	212.85 (4.64)			
Insulin (ng/ml)	CG	18.81 (0.67)	19.12 (0.57)	$p < 0.001$ (0.84)	$p < 0.001$ (0.78)	$p < 0.001$ (0.77)
	SG	18.80 (0.75)	17.60 (0.51)			
	TG	18.83 (0.40)	16.12 (0.43)			
	TSG	19.12 (0.49)	15.51 (0.55)			
Glucose (mg/dl)	CG	96.44 (13.10)	90.73 (6.43)	$p < 0.001$ (0.80)	$p < 0.001$ (0.50)	0.02 (0.21)
	SG	98.99 (10.71)	84.77 (4.50)			
	TG	99.27 (5.72)	74.08 (5.43)			
	TSG	101.63 (7.13)	71.51 (7.71)			
HOMA-IR	CG	4.48 (0.70)	4.27 (0.30)	$p < 0.001$ (0.87)	$p < 0.001$ (0.70)	$p < 0.001$ (0.44)
	SG	4.58 (0.46)	3.68 (0.25)			
	TG	4.61 (0.24)	2.94 (0.26)			
	TSG	4.79 (0.38)	2.73 (0.31)			

Abbreviations BMI: body mass index, FFM: Fat-free mass, TG: triglycerides, TC: total cholesterol, LDL: low-density lipoprotein, HDL: high-density lipoprotein

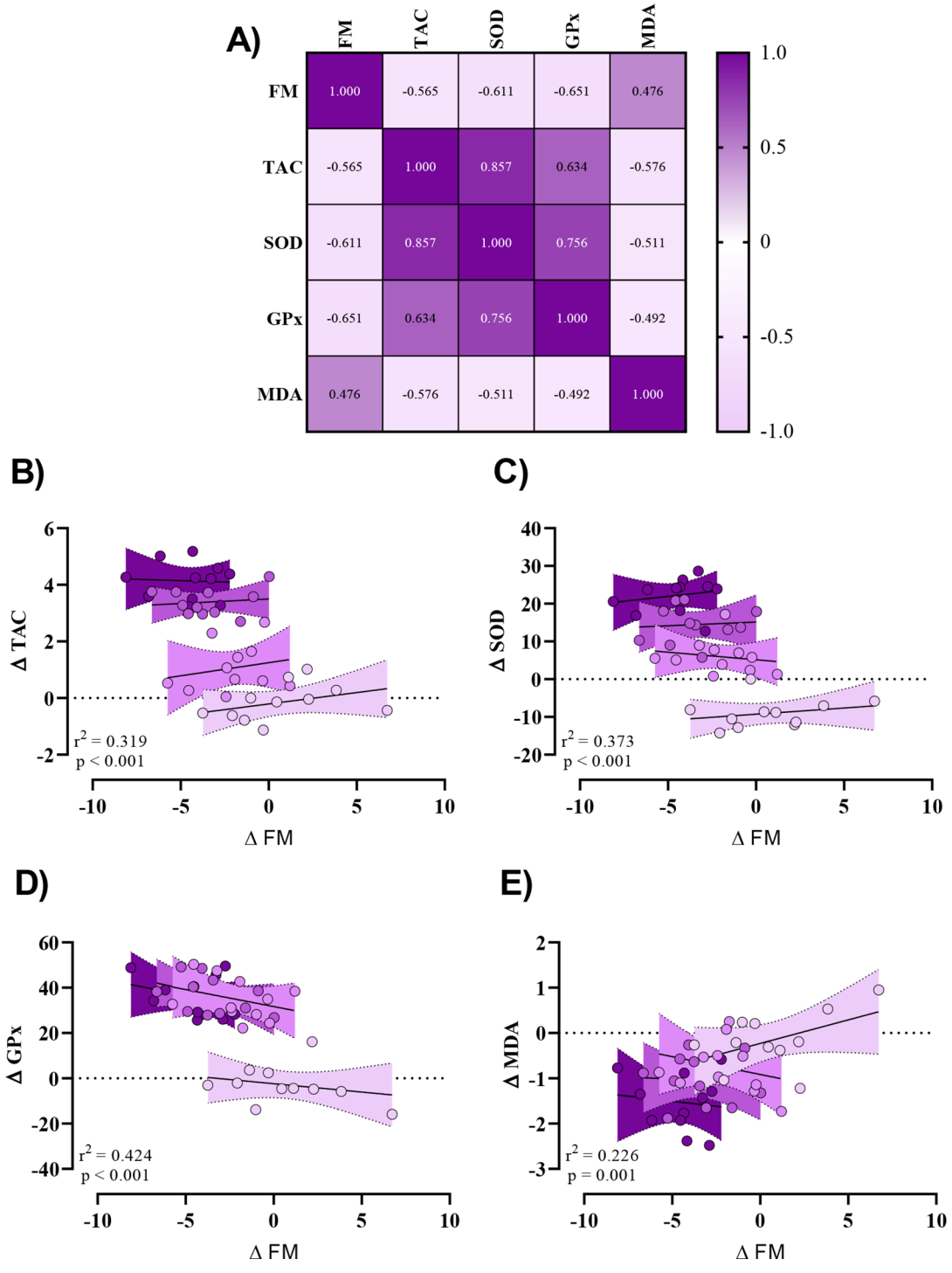

Fig. 2 (**A**) Correlation matrix of Δ FM and oxidative stress markers, r values as shown. Key indicates magnitude of r (True purple = −1 or 1, Lavender = 0). (**B–E**) linear regression (Pearson's) of Δ (marker) as a function of Δ FM (kg). Linear regression is indicated by solid black line, 95% confidence intervals are indicated by various purple colors

obese individuals, who often exhibit elevated levels of oxidative stress [55, 56]. The results from other studies were inconsistent with our results. For example, Ugras et al. reported that MDA levels increased while the activity of CAT and SOD decreased in post-training and after the championship in Muay Thai athletes [57]. Also, Sarkar et al. reported that 8 weeks of HIIT resulted in significantly increased GPx and MDA along with significant reductions in SOD in male football and hockey players [58]. The discrepant results might be due to the training

protocols, the initial training status; and the gender, age, nutritional, and health status of the subjects [57, 58]. Exercise duration and intensity are two major factors in lipid peroxidation and antioxidant response [59]. Excessive HIIT or overtraining leads to the overproduction of ROS and redox equilibrium disruption, all of which worsencellular homeostasis and impair the ability of antioxidant enzymes to scavenge lipid peroxidation [57, 58]. Well-known potential markers to assess the level of skeletal muscle damage are LDH and CK. Exercise intensity, the type of contractions, the initial muscle length and the force per active area are some of the variables that affect muscle injury [58]. The present study depicted a significant decrease in CK and LDH levels after 12 weeks of CrossFit workouts which is aligned with the fndings of the research done by de Araujo et al., where both CK and LDH decreased after 12 weeks of HIIT because of the reduced physiological stress [60]. In contrast, Bessa et al. have reported that levels of CK and LDH were raised 72 h after high-intensity aerobic training [68]. The authors attribute the raised levels of CK and LDH to their exercise protocol's sufficiently high intensity, however the levels of the antioxidant enzymes did not show any significant changes [61]. Also, the times of samples acquired after exercise were too short to change the levels of the antioxidant factors. Sarkar et al. found that markers of muscle damage showed a significant increase only after a brief period of high-intensity exercise, and not following an extended duration of low-intensity workouts [58]. Insulin, glucose, and insulin resistance, as obesity-associated factors, are major glucose homeostasis parameters to measure metabolic syndrome [62, 63]. The current research found a significant decrease in insulin, glucose, and insulin resistance which corroborates with some studies [62, 64]. For example, Jones et al. reported that 12 weeks of water-based exercise significantly reduced insulin, glucose, glucose tolerance, waist circumference, and waist-to-hip ratio in overweight women [64] and After eight weeks, Motahari-Tabari et al. found that the exercise group's fasting glucose and insulin resistance were significantly lower than those of the control group [62]. They revealed these changes were higher in people with metabolic disorders such as diabetes, cancer, and obesity [62]. Several studies found that HIIT reduced both insulin resistance and body composition or fat distribution [62, 63]. They showed that training adaptations have been linked to advantageous changes in body composition, muscle physiology, and glucose metabolism [63]. It seems that the effectiveness of insulin appears to be associated with increased skeletal muscle glucose uptake, decreased body fat and lipids level, and increased muscle cell lipid oxidative capacity [62, 63]. These observations agree with our results where we found a significant decrease in body mass, FM, LDL, and TC after 12 weeks of CrossFit workouts and in comparison to a control group.

It also appears that some adaptations are associated with the improvement of cardiorespiratory fitness, increased muscle oxidative capacity, and promoted oxygen consumption. Significant improvement in VO_{2max} after 12 weeks of HIIT was completely dependent upon changes in BMI and FFM [65]. However, in the present study, there were significant reductions in FM and FFM, while changes in BMI were not statistically significant. It seems improvements in lipid profile and cardiorespiratory responses to exercise are related to exercise dose [65]. Recently, attention to therapeutic options such as dietary supplementation has been increased by researchers [66, 67].

Numerous research papers have demonstrated that consumption of herbal antioxidants supplementations, with or without exercise can reduce oxidative stressors during some physiological and pathological processes such as different diseases [53, 68], obesity [42, 54], and high-intensity exercises [42, 54, 66, 67]. We examined the protective impacts of Spinach thylakoid extract with and without CrossFit workouts for 12 weeks in obese males. Our findings showed antioxidant indicators, muscle damage indices, glucose homeostasis parameters, blood lipids, and body composition variables improved significantly after 12 weeks of thylakoid consumption with and without CrossFit workouts. According to studies, thylakoids, which are found in chloroplasts in plants, contain antioxidant qualities. These qualities can play a critical role in regulating metabolic events, which may account for the observed protective effects [69–71].

Thylakoids taken orally have been shown to increase feelings of fullness [72], lower blood lipid levels [49, 70], lower fasting blood glucose levels [70, 73], and result in significant weight loss. In line with our results, the intake of thylakoid extracts over an 8-week period resulted a significant improvements in insulin resistance, fasting blood sugar, tumor necrosis factor-alpha,, HDL-C, TG, TC, LDL-C, and body weight among rats [71]. By scavenging ROS, the antioxidant and anti-inflammatory properties of thylakoids may be useful in reducing oxidative stress [71]. On the other hand, phenolic chemicals and galactolipids found in thylakoids have the ability to delay the digestion of fats in food. This is accomplished by blocking the action of the pancreatic lipase-colipase complex, which in turn stops the intestines from breaking down lipids (citations 71, 73). When 12 weeks of spinach thylakoid extract were given to obese PCOS female, there were significant reductions in waist circumference, weight, insulin levels, fat mass, and HOMA [49]. Also, the results of our research showed that thylakoid alone and its consumption with CrossFit workouts can improve lipid profile, increase antioxidant enzymes and reduce muscle

damage indicators. The use of some herbal supplements along with exercise can be useful to reduce the side effects and improve efficacy based on the type of training, supplements taken, and the status physical fitness of the subjects. The beneficial effect of physical training and dietary supplements, separately, on antioxidant status [66, 74–76], muscle damage [58, 60, 77, 78], physical performance [66, 67, 69, 78] and lipid profiles [62, 64, 67, 78] have been widely documented in different studies. So, the combination of these factors could be more effective as a stimulant modality [42, 54, 67, 78]. No study has been conducted on the effect of CrossFit workouts and Spinach thylakoid extract on antioxidant and oxidative stress markers in obese males, but in the available studies using the same exercise and supplementation interventions, the factors like the type of supplements, the protocol of the training, and the nutritional and physical fitness status of the subjects have been pointed out as affecting factors on the results. In line with our study, it has been shown that six weeks of CrossFit training either by itself or in combo with green tea caused an increase in VO_{2max} (7%) and SOD activity (4%). It's interesting to note that tea catechins with training may boost endurance by stimulating the consumption of oxygen by muscles, fostering the development of new mitochondria, and encouraging fatty acid β-oxidation in both skeletal muscle and overall fat utilization [39]. Additionally, green tea catechins are crucial for maintaining a balance between the body's natural antioxidant defense system and the production of ROS, which helps prevent muscle injury and enhance exercise performance. Michnik et al. [46] investigated the preventive effects of CrossFit training parallel with green tea supplementation on physical performance and biomarkers of muscle damage. Their data depicted green tea supplements improved post-training increases in CK activity and mitigated the muscle damage induced by physical exercise [38].

One of the limitations of the this research was the lack of supplement measurement based on the body weight of the subjects, so it is necessary to investigate this issue in future research.

Conclusion

In this investigation, we provided novel and important information on the advantages of combining CrossFit workout with the Spinach thylakoid extract in the regulation of some physiological parameters in obese males. We found these nondrug strategies had protective effects on antioxidant indicators (TAC, SOD, GPx, and MDA), muscle damage indices (CK / LDH), glucose homeostasis parameters, blood lipids, and body composition variables. However, the combination of CrossFit workouts with spinach-derived thylakoid has shown better effects on these factors, rather than using each of them separately. New research approaches are needed to investigate the preventive and therapeutic measures of Spinach thylakoid extract along with different types of training in different populations.

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Author contributions
Conceptualization; methodology: M.N., N.B., M.H., A.S., H.Z., M.K.H.; validation, M.H., A.S., A.A, H.Z; formal analysis, R.B., M.R., N.K., N.N., A.R.,; investigation, A.S., K.H., M.H., Y.M., F.S.; data curation Z.F., R.K., M.H., Y.M., F.S.; writing—original draft preparation, M.H., Z.S., A.A., writing—review and editing: all the authors. All authors have read and agreed to the published version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Institutional Review Board Statement
Every process followed the most recent edition of the Helsinki Declaration. The Iranian Registry of Clinical Trials (IRCTID: IRCT20151228025732N77) and the Research and Ethics Committee of the Islamic Azad University, Damghan branch (Ethics code: IR.IAU.DAMGHAN.REC.1401.034) approved the research methodology.

Informed consent
Informed consent was obtained from all subjects involved in the study.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Biomechanics and Sports Injuries, Faculty of Physical Education and Sports Sciences, Kharazmi University, Tehran, Iran
[2]Department of Sport Physiology, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran
[3]Food Science Unit, National Council for Scientific Research, Lebanon (CNRS-L), Beirut, Lebanon
[4]Faculty of Public Health, Section I, Lebanese University, Beirut, Lebanon
[5]Laboratory of Motor System, Handicap and Rehabilitation (MOHAR), Faculty of Public Health, Lebanese University, Beirut 6573, Lebanon
[6]Department of Family Medicine and Community, College of Medicine, King Faisal University, Al Ahsa, Saudi Arabia
[7]Research Center of Physiology, Department of Physiology, Semnan University of Medical Sciences, Semnan, Iran
[8]Damghan Branch, Islamic Azad University, Damghan, Iran
[9]Department of Exercise Physiology, Semnan Branch, Islamic Azad University, Semnan, Iran
[10]Department of Exercise Physiology, Faculty of Sport Sciences, University of Mazandaran, Babolsar, Iran
[11]Department of Sport Science, Faculty of Humanities, Kosar University of Bojnord, Bojnord, Iran
[12]Department of Exercise Physiology, University of Isfahan, Isfahan 81746-73441, Iran
[13]Department of Physical Education and Sport Sciences, Faculty of Humanities and Social Sciences, University of Kurdistan, Sanandaj, Kurdistan, Iran
[14]Department of Family Medicine and Public Health, Sultan Qaboos University and Sultan Qaboos University Hospital, Muscat, Oman
[15]Department of Kinesiology, Kansas State University, Manhattan, KS, USA
[16]Univ Rennes, M2S (Laboratoire Mouvement, Sport, Santé), Rennes EA 1274, F-35000, France
[17]Institut International des Sciences du Sport (2I2S), Irodouer 35850, France

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