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The Effect of Hydroxy-Beta-Methyl Butyrate Supplementation and Boxing Training on Oxidative Stress Indices in Male Boxers: A Randomized Clinical Trial

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Original Article

Abstract

Introduction: The production of free radicals during exercise is involved in the development of muscle damage and the development and spread of inflammation following exercise. Studies have confirmed the positive effect of Hydroxy-beta-Methyl Butyrate (HMB) in reducing oxidative stress indices. The purpose of this study is to compare the effect of HMB supplementation and boxing training on oxidative stress indices in male boxers.

Materials and Methods: 40 male boxers were selected through convenience sampling and were randomly divided into 4 groups (10 participants per group) of boxing training, HMB supplementation, combination, and placebo. The participants in the boxing training group performed boxing exercises for eight weeks, 3 sessions per week every other day. At the same time, the participants in the HMB supplement group consumed 7 g HMB per each kilogram of body mass. The group refrained from boxing while studying. The combined group performed boxing exercises and took HMB supplements at the same time, and the control group performed boxing exercises and placebo. Blood samples were taken to measure malondialdehyde (MDA), superoxide dismutase, and carbonyl protein 24 hours before the first day of intervention and 24 hours after the last day of intervention. The data were analyzed using analysis of covariance (ANCOVA). P value less than 0.05 was considered significant.

Results: The results showed that there was no significant difference between the effects of HMB supplementation, boxing training, and combination of HMB supplementation and boxing training on MDA in male boxers (P = 0.436). However, there was a significant difference between the effect of HMB supplementation, boxing training, and the combination of HMB supplementation and boxing training on the protein carbonyl (P = 0.02). The mean of protein carbonyl in the boxing athletes with supplement was significantly better than the other groups (P = 0.013).

Conclusion: In general, due to the effectiveness of HMB supplementation and boxing training on some indicators of oxidative stress, supplementation with the appropriate dose is recommended to athletes.

Keywords: HMB Supplement; Boxing training; Malondialdehyde; Protein Carbonyl

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Introduction

Boxing is an intermittent sport with intense explosive activity, and these intense explosive activities may cause lactic acidosis, muscle fatigue, and eventually muscle damage (1). Depending on the type, intensity, and duration of physical activity, a wide range of changes occur in the body of boxing athletes, including increased production of free radicals, damage to body tissues, production of stress hormones, changes in the number of macrophages, neutrophils, and neutrophils, decreased immune activity and, ultimately, an increased risk of infection (2). Free radicals are species with very short half-lives and very strong reactivity, which are neutralized by a precise antioxidant defense system consisting of enzymes such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX), and anti-GPX, and antioxidants such as C, E, and A vitamins, carotenoids, glutathione, flavonoids, and selenium (1). Oxidative damage produces products such as

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malondialdehyde (MDA) and carbonyl protein, which are known as lipid peroxidation index (3). Oxidative damage also produces products of MDA and SOD, which are known to be the fat damage indices. MDA is an aldehyde, active, and highly reactive compound, produced in the human body by the peroxidation of unsaturated fatty acids. In contrast, SOD is the most important enzyme that decomposes superoxide radicals in the human body (4).

There is ample evidence that exercise improves the antioxidant defense system (1-3). Therefore, it protects skeletal muscle from damage caused by free radicals after exercise (5,6). In addition to exercise, which somewhat improves the antioxidant defense system, another way to strengthen the antioxidant system is to use supplements (1). The results of some previous studies indicate that the use of nutritional factors and dietary supplements can be one of the appropriate strategies to prevent cell damage and increase in inflammatory markers (1,6,7). The Beta-Hydroxy-beta-Methylbutyrate (HMB) supplement has been considered by athletes for nearly two decades. HMB is a branched-chain amino acid metabolite (BCAA) of leucine and its ketoacid (8). HMB appears to have an anticatabolic effect in reducing muscle protein breakdown (MPB) and exercise-induced injury. Although there is no direct evidence for the mechanism of action of HMB (9), it is hypothesized that HMB acts as a precursor to cholesterol synthesis by metabolizing it to beta-hydroxy-betamethyl CoA and thus providing a carbon source (10). Another hypothesis is that HMB is beneficial for the structural part of the cell membrane. Numerous studies have advocated the efficacy of HMB supplementation in increasing and improving lean body mass (LBM), creatine kinase, and strength, lowering low-density lipoprotein (LDL), and increasing anaerobic power (ANP) and function (8,9). The results of studies on trained martial artists and professional rowers show that taking HMB supplementation has a significant effect on increasing LBM, reducing body fat, increasing ventilatory threshold level, heart rate threshold, ANP, mean power, and maximum speed compared to the placebo group (11,12).

Studies have been conducted on the effects of HMB supplementation on cell damage markers, with some confirming the positive effect of HMB in reducing the symptoms of muscle injury. In his study, Muller examined the effect of HMB supplementation on body composition and muscle power production in non-competitive male athletes aged 19 to 24 years. These men did resistance training three times a week for 8 weeks; In thin study, creatine kinase (CK) activity decreased (13). Nevertheless, other studies have yielded conflicting results and have shown that this supplement does not reduce the risk of muscle injury. In a study, Arazi et al. examined the effect of HMB supplementation and resistance training on oxidative stress indices and concluded that the HMB supplementation along with resistance training did not significantly affect oxidative stress markers (MDA) (14). In another study by Arazi et al., the effect of HMB supplementation and resistance training on cardiovascular risk factors and oxidative stress was investigated, with the results suggesting that the short-term use of HMB supplementation along with resistance training did not affect the cardiovascular risk factors and oxidative stress index (OSI) (MDA) (15). In a study, Sheikholeslami Vatani and Ahmadi examined the effect of acute use of HMB and creatine supplements on oxidative and antioxidant indices in subjects who performed resistance exercise and concluded that creatine, HMB supplementation, or a combination of both did not affect the MDA level, GPX antioxidant changes, bilirubin, and uric acid (8). In other words, the effects of the HMB supplementation may vary depending on the exercise. Additionally, in athletes, the effects of the HMB supplementation on longer protocols seem to be optimized (16).

The outcomes of various studies indicate that the HMB supplementation may elicit different antioxidant responses depending on the type, duration, and intensity of exercise, as well as the dose and loading period during different post-workout periods. Due to the fact that by searching the databases, no study was found in this field using boxing exercises, in the present study, the effect of HMB supplementation and boxing training on performance and some antioxidant indices in male boxers was compared.

Materials and Methods

This study was a clinical trial performed as a pretest-posttest with three experimental groups and a control group (placebo) in 2019. After announcing the call to participate in the study, among the volunteers participating in boxing classes in Takhti gym of Dolatabad City, Iran, and people with three years of boxing experience, 47 male boxers volunteered to participate in the study. Among these athletes, 40 people were selected and using a table of random numbers (to use random numbers, the researcher first determined the direction of reading of the table numbers, then put his hand on one of the numbers and moved in one of the preset directions and recorded

the numbers and assigned them to different groups), were randomly assigned into the four groups of boxing training (n = 10 people), HMB supplementation (n = 10), combined intervention [boxing training and HMB supplementation (n = 10)], and control group [Placebo (n = 10)].

The study inclusion criteria were athlete satisfaction, no smoking, no cardiovascular diseases (CVDs), hypertension, respiratory diseases, and musculoskeletal disorders (MSDs) (based on the athlete self-report). Withdrawal from the project, absence on the day of the study, and participation in heavy sports training other than boxing were also considered as the exclusion criteria, which were examined by the researcher. In a session, the participants were informed about the type of study, study objectives and implementation method, and benefits and potential risks, and informed consent was obtained from them. The study protocol was approved by the Ethics Committee in Biomedical Research, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran.

Prior to the start of the project, a preliminary session was held 48 hours before the start of the interventions in the Takhti gym of Dolatabad. In this session, after explaining the objectives of the study, to measure the anthropometric index, the weight and height of the subjects were measured by a digital scale (MW86-E, SNOWA, Iran) with an accuracy of 0.1 kg and a stadiometer (217, Seca, Germany) with an accuracy of 0.5 cm, respectively. Then, the body mass index (BMI) was calculated by dividing weight in kilograms by square height in meters.

Blood sampling was performed in two stages: before the first session and 48 hours after the last session in the eighth week, in the medical diagnostic laboratory and after 12 hours of fasting and at rest from the left hand vein of the subjects at a rate of 10 ml in the sitting position. The blood samples collected were placed in sterile tubes and the serum was separated by centrifugation at 4 °C for 15 minutes at 2000-3000 rpm and the samples were kept at -70 °C until measurement. The subjects were asked to refrain from any exercise for 24 hours prior to blood sampling. After collecting in the post-test stage, all blood samples were tested in one day.

HMB supplement use: In the present study, 3 grams of HMB (Clear Muscle, 0000853250, MuscleTech, USA) was consumed daily as three 1 gram meals per day (morning, noon, and night) for 8 weeks based on the agreement in previous studies (13,14). In the placebo group, the subjects consumed dextrin (Dextrose, Iran). The placebo was indistinguishable in appearance and taste and was offered as a supplement. The placebo package was placed in the same manner as that of HMB. The participants were required to return all used and unused packages at the end of each week. Reminding of the consumption was performed daily by the researcher via the text message (17).

Boxing training program: included 8 weeks and a total of 32 boxing training sessions that were designed in three stages (phases) as presented in table 1.

Table 1. Boxing exercises (17)

Variable and phase	First phase (basic)	Second phase (special)	Third phase (tapering)
Duration of training (weeks)	2	3	3
Number of sessions per week	4	4	4
Number of rest days per week	3	3	3
Practice time per session (minutes)	110	100	90
Exercise intensity (maximum heart rate) (%)	70	80	90

The first phase of training included the overall development of physical fitness components (such as strength, power, agility, endurance), which was named as the basic phase. These exercises consisted of 2 weeks and 4 sessions of 110 minutes per week, which were performed with an intensity of 70% of the maximum heart rate. In order to control the intensity of training, a Polar pacemaker (Polar, Finland) was utilized.

The second phase of the training included the special development of physical fitness components and the increase of advanced technical skills, which was named as the special phase. These exercises consisted of 3 weeks and 4 sessions of 100 minutes each week, which were performed with 80% intensity.

The third phase of the training included tactical and technical performance, training for the main competition in addition to emphasis on competitive and tactical training, which was named as the tapering phase. These exercises included 3 weeks and 4 sessions of 90 minutes per week, which were performed with 90% intensity.

The control group practiced boxing and placebo. Accordingly, all groups except the HMB group participated in the boxing exercises during the study as described.

Biochemical measurements: to prepare the serum,

blood samples were collected from the subjects' antecubital vein as 10 cc in a specialized laboratory after 12 hours of fasting from 8-11 am in two stages (the first stage 24 hours before the start of boxing training and taking HMB supplement and the second stage 48 hours after eight weeks of boxing training and consumption of HMB supplement). The blood collected was transferred to sterile tubes and the serum was separated by centrifugation (12, Dlasent, UK) at 4 °C at 2000-3000 rpm for 15 minutes and the samples were kept at -70 °C at Dr. Baradaran medical diagnostic laboratory until measurement. The subjects in the experimental group were asked to abstain from any exercise 24 hours before blood sampling. To separate serum from plasma, the samples were placed at laboratory temperature for 30 minutes and then centrifuged for 5 to 10 minutes at a rate of 2000 rpm. All measurements were performed by a laboratory expert who was unaware of the subjects' condition. MDA in the present study was measured using a kit (Cayman Chemical, USA). The kit was based on chemical colorimetry and the basis of the measurement was the reaction between MDA with thiobarbituric acid and the formation of a color complex. The sensitivity of the method used was 0.08 µmol and the intratest coefficient of variation was determined as 5.9%. The unit of measurement was nanomoles per milliliter. In the present study, carbonyl protein was determined by measuring the serum levels of the carbonyl protein enzyme and SOD by the chemical colorimetric method and using the kit (Cayman Chemical, USA) in terms of nanomoles per milliliter.

Data analysis was performed at both descriptive and inferential levels. At the descriptive level, the mean and standard deviation (SD) values were used to describe the condition of the sample and at the inferential level, the analysis of covariance (ANCOVA) test was employed to compare the effect of the intervention among the four groups, while controlling the effect of the pretest score. Before performing the tests, the assumptions of the normal distribution of the samples and the homogeneity of variance of error between the two groups were tested using the Shapiro-Wilk and Levene tests, respectively. The collected data were analyzed in SPSS software (version 21, IBM Corporation, Armonk, NY, USA). $P < 0.050$ was considered as the significant level.

Before conducting the study, the necessary coordination was made with the boxing board of Isfahan Province, Iran. Before the start of the test, all subjects were examined by a physician and received permission to participate in the test. Moreover, explanations about the study process were provided to the subjects and written consent was obtained from them. The present study was approved with the ethics code IR.IAU.KHUISF.REC.1398.055 in the ethics committee of Isfahan (Khorasgan) Branch, Islamic Azad University and was registered in the Iranian Registry of Clinical Trials (IRCT) system with the code IRCT20170510033909N4.

Results

In the present study, there was no dropout of the participants and all of them completed all stages of the study. Accordingly, it was not possible to perform an intention-to-treat (ITT) analysis (Figure 1).

The mean demographic indicators of the subjects are presented in table 2. Examination of the homogeneity of the groups using the one-way ANOVA test demonstrated that the four groups were homogeneous in the variables of age, height, and weight ($P > 0.050$). Table 3 presents the mean values of the variables and the ANCOVA results.

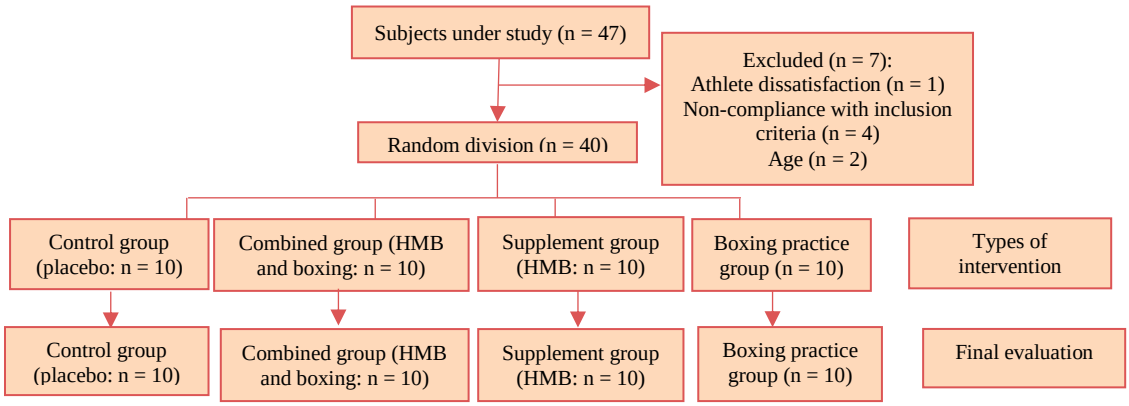

Figure 1. Consolidated Standards of Reporting Trials (CONSORT) flowchart of the study process and the dropout of subjects during it

HMB: Hydroxy-beta-Methyl Butyrate

Table 2. Mean demographic characteristics of the subjects in the four groups

Group	Age (year)	Height (cm)	Weight (kg)	BMI (kg/m2)
Control	21.10 ±2.13	175.90 ±2.73	75.40 ± 7.43	24.40 ± 0.80
Boxing	22.30 ± 1.42	175.00 ±3.53	75.00 ±5.77	24.50 ± 0.60
Supplement	21.70 ± 2.06	174.90 ± 3.70	75.40 ±4.35	24.45 ± 0.72
Combined intervention	21.50 ±1.78	174.50 ±3.24	75.20 ± 4.02	25.00 ± 0.90
F statistic	0.717	0.317	0.012	1.400
P	0.549	0.813	0.998	0.781

BMI: Body mass index

Based on the data in table 3, by controlling the effect of pre-test scores, a significant difference was observed in the carbonyl protein variable among the four groups in the post-test stage (P = 0.020). The results of the Bonferroni post hoc test indicated that the mean amount of carbonyl protein in the boxing athletes group was significantly higher than that in the combined boxing and HMB group (P = 0.013). In other pairwise comparisons, no significant difference was observed between the groups (P > 0.050). Furthermore, by controlling the effect of the pre-test scores, there was a significant difference among the four groups in the maximum oxygen consumption variable in the post-test stage (P = 0.018). The results of the Bonferroni post hoc test suggested that the mean maximum oxygen consumption in the control group was significantly lower than that in the boxing and HMB groups (P = 0.025), however it did not show a significant difference with the maximum oxygen consumption in the boxing (P = 0.132) and supplement (P = 0.074) groups.

Given the results of table 3, there was no significant difference in the variables of MDA (test power = 0.238, P = 0.436), SOD (test power = 0.245, P = 0.480), and resting heart rate (test power = 0.549, P = 0.085) among the four groups. Besides, the results of the paired t-test showed that the intra-group changes in MDA, SOD, and resting heart rate variables from pre-test to post-test were not significant.

Discussion

The aim of this study was to compare the effect of HMB supplementation and boxing training on oxidative stress indices in male boxers. The boxing and combined intervention groups performed boxing exercises for eight weeks.

Table 3. Mean and results of analysis of covariance (ANCOVA) in comparison of scores of athletes in four groups in the post-test stage

Variable	Group	Pre-test	Post-test	Intra-group difference	Inter-group difference			
					F statistic	P	Eta squared	Test power
Serum MDA level (nanomoles per milliliter)	Control	40.50 ± 6.02	39.60 ± 6.35	0.753	0.931	0.436	0.074	0.238
	Boxing	43.80 ± 6.58	43.00 ± 8.65	0.763				
	Supplement	39.70 ± 6.18	41.020±5.87	0.600				
	Combined intervention	39.10 ± 7.39	44.80 ± 7.84	0.233				
Serum SOD level (SI unit per liter)	Control	5.89 ± 0.16	5.95 ± 0.14	0.286	0.842	0.480	0.067	0.245
	Boxing	5.88 ± 0.16	5.92 ± 0.22	0.753				
	Supplement	5.88 ± 0.16	5.92 ± 0.14	0.485				
	Combined intervention	5.83 ± 0.15	6.04 ± 0.21	0.099				
Carbonyl protein serum level (ng/ml)	Control	1.61 ± 0.06	1.62 ± 0.05	0.916	30716	0.020	0.242	0.760
	Boxing	1.62 ± 0.04	1.66 ± 0.07	0.214				
	Supplement	1.61 ± 0.04	1.61± 0.05	0.831				
	Combined intervention	1.62 ± 0.06	1.58 ± 0.04	0.042				
Control heart rate at rest (number per minute)	Control	66.40 ± 4.20	66.30 ± 3.80	0.963	2.394	0.085	0.170	0.549
	Boxing	67.40 ± 2.80	65.10 ± 3.11	0.067				
	Supplement	66.20 ± 4.18	69.10 4.31±	0.208				
	Combined intervention	68.00 ± 4.06	65.00 ± 3.53	0.069				
Maximum oxygen consumption (ml/kg/min)	Control	39.80 ± 2.30	39.10 ± 3.11	0.651	3.723	0.018	0.247	0.793
	Boxing	38.70 ± 3.23	42.60 ± 2.80	0.049				
	Supplement	39.80 ± 2.30	42.60 ± 3.69	0.059				
	Combined intervention	40.10 ± 2.69	43.10 ± 2.23	0.029				

During this time, the HMB supplement and placebo groups performed their daily activities, and the other groups consumed 3 grams of dextrin supplement/placebo per one kilogram of their body weight a day for eight weeks. Blood samples were taken 48 hours before and after the start of the study and the results suggested that eight weeks of HMB supplementation had no significant effect on the MDA index, which was consistent with the findings of the study by Arazi et al. (13). One of the possible reasons is the same dose of supplementation in both studies. Sheikholeslami Vatani and Ahmadi showed that creatine and HMB supplementations or a combination of both had no effect on the MDA index (8). Improving the antioxidant system of individuals who practice regularly and long-term, is involved in maintaining the oxidation system and cell regeneration (3); This is because long-term aerobic exercise, in addition to increasing the capacity to produce aerobic energy in the muscle, also improves the antioxidant status of the muscle. On the other hand, the results of the study by Tuna et al. illustrated that the concentration of non-enzymatic antioxidants increases with aerobic exercise (6). Frequent production of free radicals due to ischemia and redistribution of blood at the muscular level (which occurs as a result of this type of exercise) seems to play a role in improving the antioxidant profile (18).

By increasing hormones such as catecholamines, prostanoids, and macrophage activity, intense and irregular physical activity affects the oxidative function of cells and cell membrane structure, increasing oxidative stress and lipid peroxidation. Decreased localized blood flow at the beginning of physical activity in organs such as active muscles, kidneys, and liver is another factor in the process of increasing lipid peroxidation; however, regular and continuous exercise, by increasing antioxidant defense, reduces lipid and protein peroxidation (19).

The findings of the present study indicated that eight weeks of boxing training had no significant effect on SOD and was in contradiction with the findings of the study by Ahmadi Kakavandi et al., who examined the effect of resistance training on SOD in the elderly (20). In their study, exercise was associated with an increase in SOD (20). One of the possible reasons for the inconsistency of the results is the type of subjects and their significantly higher age in the study of Ahmadi Kakavandi et al. (20) compared to the present study. Hormonal responses to resistance training depend on factors such as duration and type of training, genetic background, gender, nutrition, age, circadian cycle, and physical fitness level of individuals (21). The amount of muscle mass involved in activity, intensity and volume of training, age, and training experience independent of the amount of muscle strength are among the factors affecting the testosterone hormone response (22).

In their study, Azizbeigi et al. compared the effect of endurance, resistance, and combination training in untrained men and reported that all three types of training led to a significant increase in SOD activity in erythrocytes (23). Free radicals, which are permanently produced by hemoglobin as a result of autoxidation inside red blood cells and continuously expose red blood cells to oxidative stress, are removed from the environment by the SOD enzyme (24).

However, the SOD enzyme is the first line of defense against the attack of free radicals and reactive oxygen species (ROS), and it seems that this enzyme is also affected by this issue. Increased SOD enzyme activity following regular exercise may be due to mitochondrial respiratory chain regulation (25). However, it seems that some other enzymes involved in energy production are also involved in adaptation to exercise (20). Following the performance of sports exercises and ischemia-reperfusion phenomenon during resistance training, the activity of the complex IV of the electron transport chain increases compared to the I-III complexes. ROS is produced in steps 1-3, but in step 4 there is a powerful antioxidant called cytochrome that recycles ROS and, by transferring electrons to oxygen, produces water, reducing electron emission and, consequently, reducing ROS production and reducing electron leakage (25). The results of the present study demonstrated that eight weeks of HMB supplementation had no significant effect on SOD index. Searching in databases, no study was found on the effect of HMB supplementation on SOD index.

Given the outcomes of the current study, eight weeks of boxing training with HMB supplementation had a significant effect on reducing the carbonyl protein index. Although no study was conducted to find out the effect of the HMB supplementation on carbonyl protein index, it seems that performing a session of intense exercise such as boxing can be associated with ROS-nitrogen production and oxidative stress damage (26). The results of a study showed that one year of aerobic exercise (30 to 45% VO_{2max} for 45 minutes five days a week) in women resulted in a non-significant reduction in urinary F2-Isoprostane (F2-IsoPs) indicating oxidative stress damage (lipid peroxidation) (27). ROS production occurs through the leakage of unpaired electrons into the electron transport chain of the inner mitochondrial membrane of the

contracting muscle cells (28). However, a combination of enzymatic and non-enzymatic antioxidants that neutralize free radicals and reduce oxidative stress can be affected by exercise. Moreover, the products of oxidative stress vary depending on the cell components, and more detailed investigation of this issue requires further investigations.

Limitations

In the present study, it was not possible to accurately control all the influencing factors including sleep and rest rate of the subjects (29). Additionally, variables such as waist circumference, hip circumference, and duration of boxing of the subjects were not recorded; While access to this information before and after the end of the study, could help analyze the effects observed. One of the important limitations of the present study was the impossibility of verifying the claim of the HMB group in not participating in the boxing training program for 8 weeks and also following the boxing group and the combined group of the desired training instructions. Not participating in the boxing program, not changing the program in the control group, and strict observance of the training program instructions in the boxing group and the combined group were considered only based on the reports of the participants. However, in the briefing before the start of the study, the importance of honesty in the reports was emphasized to the participants and during the study, their adherence to the program was monitored via text message and phone call. On the other hand, the 8-week suspension of the HMB group from boxing exercises can be a factor for the possible analysis of this group during the study if the members of this group fully adhered to the desired protocol, which needs to be considered in interpreting the results.

Recommendations

Given the importance of other oxidative stress indicators during and after intense exercise, it is recommended to measure, for example, the total antioxidant capacity (TAC) in future studies. Due to the fact that not much research has been accomplished on antioxidant enzymes (such as catalase and GPX), it is suggested that a study be conducted to investigate the effect of HMB supplementation and aerobic activity on antioxidant enzymes. Furthermore, due to the lack of definite effects of age, sex, and other variables related to environmental stressors, it is recommended that the effects of such variables be examined on the indicators studied in the present study. Besides, studying the effect of some medicinal plants with antioxidant properties can yield valuable results.

Conclusion

The present study considered the course of HMB supplementation to be eight weeks and the dose to be 3 grams, which may not have an effect on oxidative parameters. Therefore, it is suggested that researchers use a longer loading period and use of these supplements to provide credible evidence to recommend the use or non-use of this supplement for boxing athletes.

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Authors' Contribution

Mehd Fatahi: study design and ideation, attracting financial resources for the study, study support, executive, and scientific services, providing study equipment and samples, data collection, exercise, analysis and interpretation of results, manuscript preparation, specialized evaluation of the manuscript in terms of scientific concepts, approval of the final manuscript to be submitted to the journal office, responsibility for maintaining the integrity of the study process from the beginning to publication, and responding to the referees' comments; Farzaneh Taghian: study design and ideation, providing study equipment and samples, data collection, exercise, analysis and interpretation of results, manuscript preparation, specialized evaluation of the manuscript in terms of scientific concepts, approval of the final manuscript to be submitted to the journal office, responsibility for maintaining the integrity of the study process from the beginning to publication, and responding to the referees' comments.

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Conflict of Interest

The authors did not have a conflict of interest. The first author provided the funding of the study.

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