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The Attenuating Effect of High-Intensity Interval Training and Nicotinamide Mononucleotide Supplementation on Aging-Related Oxidative Stress in Rat's Heart Tissue

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Original Article

Abstract

Introduction: Numerous studies have shown attenuating effects of nicotinamide mononucleotide (NMN) and high-intensity interval exercise (HIIT) training on reducing oxidative stress. Therefore, in the present study, we evaluated the effect of HIIT and NMN supplementation on the oxidative stress markers in cardiac tissue of rats.

Materials and Methods: 48 sprago dauli rats with an average age of 8 to 10 months were randomly divided into 6 groups of pretest control, control, sham (normal saline), HIIT, NMN supplementation, and combination (HIIT + NMN). The HIIT and combination groups performed HIIT on the treadmill for 8 weeks, 3 sessions per week. NMN with a dose of 500 mg per kilogram body weight was administered intra-peritoneally in the NMN and combination groups. 24 hours after the last training and NMN administration, the animals were sacrificed, and their hearts were removed for evaluating oxidative stress factors [malondialdehyde (MDA), protein carbonyl (PC), superoxide dismutase (SOD), and glutathione peroxidase (GPx)]. One-way ANOVA test was used for statistical analysis.

Results: The expression of GPx gene in the groups receiving NMN, HIIT, and combination was significantly higher compared to the pretest control, control, and sham groups ($P < 0.050$). Moreover, the levels of MDA and PC were lower in NMN, HIIT, and combination groups compared to the pre-test control, control, and sham ($P < 0.050$). The highest reduction was seen in the combination group ($P < 0.001$).

Conclusion: It seems that regular consumption of NMN and/or HIIT may attenuate oxidative stress in the cardiac tissue; however, combinations of NMN and HIIT significantly improves the effects of sole interventions.

Keywords: Nicotinamide mono nucleotide; High-intensity interval exercise; Oxidative stress

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Introduction

Accumulation of various harmful changes in cells and tissues occurs following aging, which are responsible for the increased risk of disease and mortality (1). Researchers have considered the increase of free radicals as one of the most important theories of the aging process. Given this hypothesis, with age, cell function, following an increase in oxidative stress, causes damage to intracellular macromolecules such as membrane fats, deoxyribonucleic acid (DNA), and proteins. Additionally, it seems that the increase of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to a level beyond the natural tolerance and capacity of the cell, in all cells that supply aerobic energy, causes the combination of these factors with protein and phospholipid structures and increased factors such as malondialdehyde (MDA) and protein carbonyl (PC), thus causing disorders in the cell by damaging intracellular organelles (2,3).

In general, endogenous sources of reactive oxygen nitrogen species (RONS) include the nicotinamide mononucleotide (NMN) oxidase, myeloperoxidase

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(MPO), lipoxygenase, and angiotensin II. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the major source of peroxide anion radical production, which is created by the reduction of one electron from an oxygen molecule by electrons produced by NADPH during cellular respiration and is eventually converted to hydrogen peroxide. Normally these free electrons are neutralized by antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) (2,4). However, with age, these antioxidant enzymes lose their effectiveness and this system is not able to compensate for all the free radicals produced. This process eventually leads to cell damage and the initiation of mitochondrial and nuclear DNA mutations (5). Moreover, the incidence of cardiovascular diseases (CVDs) increases with age; So that heart failure in individuals over 85 years is four times higher than that in people 65 to 75 years (6). Numerous studies have investigated the effect of high intensity interval training (HIIT) on oxidative stress and antioxidant status (7-10). Research has shown that HIIT can reduce oxidative stress and improve antioxidant status. Besides, in patients with heart failure, GPX increased following HIIT (11). Moderate-intensity interval training (MICT) has a significant increase in antioxidants compared to HIIT (12) and has stronger positive effects on cardiorespiratory function, risk factors for CVDs, and markers related to vascular function (13). Recent studies have indicated that long-term physical activity can slow down the aging process by affecting chromosomes (14,15). In addition to physical activity, taking appropriate dietary supplements also contributes to improving the body's antioxidant status (7).

Recently, strong evidence has revealed that intracellular nicotinamide adenine dinucleotide (NAD) biosynthesis with dietary supplements can help increase organ lifespan and overall cell health (16). Evidence suggests that lower NAD levels shortens cell life; as the decreased levels of this index inhibit the expression pathway of Sirtuin 1 (SIRT1) and by inhibiting downstream signaling of mitochondrial biogenesis, lead to impaired cellular respiration, reduced longevity of intracellular organs, and reduced telomere length (17). NMN treatment improves peripheral vascular function and increases cellular respiration (18). In fact, NMN is a nucleotide derived from ribose and nicotinamide and is made from B vitamins in the body and is naturally present in all forms of life (19). Nowadays, researchers are looking for a way to combine exercise with supplements or herbs to gain more favorable effects in preventing the reduction of oxidative stress.

Despite numerous studies, no study was found to examine the interactive effect of interval training with NMN supplementation on reducing oxidative stress in cardiac tissue. Since the adverse effects of aging on heart health are increasing dramatically worldwide, the number of elderly people who suffer from debilitating diseases due to lack of exercise is expected to increase in the coming decades. Therefore, it would be worthwhile to conduct a study to investigate the interactive effect of interval training and NMN supplementation on reducing oxidative stress in heart tissue.

The study of cardiac markers and the expression of genes, given the ethical considerations, requires the use of animal models. Rats are a type of laboratory animals that are used as a good model of humans in the study of the cardiovascular system. The general findings show that mice grow rapidly in childhood and reach sexual maturity around the sixth week, but reach social maturity 5-6 months later. Each day of the animal's life in adulthood is approximately equivalent to 34.8 human days (i.e., one month of a mouse's life is comparable to three years of human life) (20). Therefore, in the present study, the effect of HIIT and NMN supplementation on oxidative stress in rat heart tissue was investigated.

Materials and Methods

Animals: In this experimental study, 48 8-month-old male Sprague-Dawley (SD) rats weighing 220 ± 20 g were selected from the Laboratory Animal Care Center, Marvdasht Branch, Islamic Azad University, Shiraz, Iran and were randomly divided into six groups.

1- Pretest control: Animals in this group were sacrificed at the beginning of the experiment.
2- Control: Animals in this group were sacrificed at the end of the experiment to investigate the effect of aging on dependent variables.
3- Sham (placebo): To determine the possible effect of the injection process, the samples of this group received 500 mg of normal saline solution [phosphate buffered saline (PBS)] (0.9 injectable sodium chloride solution) per kilogram of body weight as an inactive substance intraperitoneally five days a week (every day except Thursdays and Fridays) for 8 weeks.
4- HIIT: To determine the net effect of HIIT, the animals were trained for 8 weeks, five days a week (every day except Thursdays and Fridays).
5- Supplement (NMN): To determine the net effect, the animals in this group received 500 mg per kg of body weight NMN supplement intraperitoneally five days a week for 8 weeks (21).
6- Combination: These animals experienced HIIT and

intraperitoneal injection of NMN supplement for 8 weeks, five days a week. The exercise was performed for all animals between 9 and 10 AM and after 2 to 3 hours, peritoneal injection was performed.

Since the aim of the present study was to evaluate the oxidative stress due to the passage of time, two control groups were considered, and to examine the effect of age, the animals of the pretest control group were sacrificed in the first week and before the start of the study and the animals of the control group in the last week at the same time as the other groups.

The rats were kept in polypropylene cages at 23 ± 2 °C and 40 to 50% humidity with a 12-hour light/dark cycle and were given standard food and drinking water with free access. The study was conducted according to the guidelines of the Iranian National Committee for Ethics in Biomedical Research (INCEBR) for the Use of Laboratory Animals.

Chemicals

NMN was purchased from Xi'an Prius Biology Engineering Co. Ltd (China, MF: C11H15N2O8P, EINECS No.:214-136-5, purity: 99%). All chemicals and other reagents used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and PBS (0.9% injectable sodium chloride solution) from Shahid Ghazi Pharmaceutical Company (Tabriz, Iran).

Study design

A. Calculation of maximum speed: The average maximum speed of the animal to determine the maximum oxygen consumption was calculated based on the incremental test of Bedford et al. (22). To calculate the maximum oxygen consumption, a treadmill for rats (Bionic Mobin Technical Engineering Company, Tehran, Iran, product code: 32588) was used. The rats first warmed up at 10 meters per minute for five minutes and then ran at 12 meters per minute for three minutes. Then, for every three minutes, the animals' speed of running increased 2 meters per minute until the rats became exhausted (exhaustion refers to a condition in which the rats hit the end of the treadmill conveyor belt three times in a row in less than a minute and were no longer able to continue the training). The treadmill slope was set to zero at all stages. At each stage of the experiment where the animal was no longer able to continue the training, the speed at that stage was considered equal to the animal's speed at the maximum oxygen consumption state (22).

B. Exercise program: The HIIT protocol included three parts of warm-up (5 minutes), exercise including 2-minute interval repetitions (2 × 2) (with an active recovery period of 2 minutes after each interval) and cooling down (5 minutes). The rats were first warmed up to 50 minutes with 50 to 60% of the maximum intensity for 5 minutes. The interval training consisted of a combination of high-intensity and low-intensity interval repetitions. The high-intensity interval repetition included 2 minutes with 80% maximum intensity in the first week, 90% maximum intensity in the second week, 100% maximum intensity in the third week, and 110% maximum intensity from the beginning of the fourth week to the end of the training, and the low-intensity interval repetition (recovery interval) consisted of 2 minutes with a maximum intensity of 50% (Figure 1).

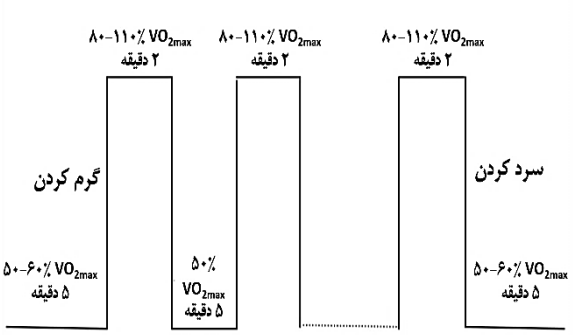

Figure 1. Schematic view of the high intensity interval training (HIIT) protocol

This program was designed based on the training protocol used in previous studies (22) on rats with metabolic disorders and the exercises were planned based on aerobic capacity and ability of rats to perform the exercise, which was examined weekly. Evidence shows that if the recovery time between the intense intervals decreases, the contribution of glycolysis to energy supply decreases and, as a result, aerobic metabolism increases to compensate for this energy loss. Aerobic metabolism during HIIT recovery periods plays an important role in the regeneration of creatine phosphate and the oxidation of lactic acid (lactate removal) (23). In other words, HIIT shifts the metabolism to aerobic and increases its capacity (23).

After the last repetition of the high-intensity interval, the rats were cooled for 5 minutes at 50 to 60% of maximum speed. The number of high-intensity interval repetitions was determined based on the training week of the rats; 2 repetitions of high intensity interval in the first week, 4 repetitions of high intensity interval in the second week, 6 repetitions of high intensity interval in the third week, and 8 repetitions of high intensity interval from the

beginning of the fourth week onwards. Hence, the total training time included high-intensity and low-intensity interval repetitions with warm-up and cooling on average 16, 24, 32, and 40 minutes in the first week, second week, third week, and fourth week onwards, respectively (18). In addition, for NMN supplementation, given the weight of the rats, first 2 g of the supplement was dissolved in 4.8 cc of PBS and then 500 mg of the supplement per kilogram of body weight (0.3 cc of solution) was injected to the samples peritoneally with a 1-cc syringe (Needle No. 2) (21). Since the injections had to be given daily and daily anesthesia caused side effects and interfered with the study results, on injection days, two to three hours after the exercise, the rats were completely restrained and the solution was gently injected into the peritoneum using the size 2 needle.

Sample collection: 24 hours after the last administration, the animals were anesthetized using a mixture of ketamine 10% (Bremer Pharma, Germany) (100 mg/kg) and xylazine (Kela, Belgium) (10 mg/kg) and were sacrificed by braking their necks and then dissected. First, the animal's chest was opened and the heart was immediately removed, and its weight was recorded using a digital scale (Sartorius, CPA224S, Germany). The heart was divided into two parts in the middle; One part was used to estimate the biochemical parameters of the tissue, including MDA and PC, and the other part was immediately placed at -80 °C for testing for gene expression and taken out of the freezer at the time of testing.

Estimation of tissue biochemical indices: The heart tissue was homogenized (10% by weight/volume) in the cold PBS (Cat No: DB0011, DNAbiotech Co., Tehran, Iran) 50 mM (pH = 7.4) and then centrifuged at 3000 revolutions per minute (rpm) at 15 °C for 15 minutes and the supernatant was used to measure biochemical parameters. The protein concentrations were obtained using the instructions given by Bedford et al. (22).

Measurement of lipid peroxidation of the heart tissue: To evaluate lipid peroxidation, the MDA level was measured. Thus, to one ml of homogenized 10% by weight/volume of heart tissue (in PBS), 0.5 ml of 10% trichloroacetic acid [CAS No: 76-03-9, Catalog No: T6399, Molecular formula: C2HCl3O2, Sigma-Aldrich (St. Louis, MO, USA)] was added and centrifuged at 3000 rpm for 15 minutes. 1 ml of 0.67% thiobarbituric acid [Catalog No: 7-52-67, Molecular formula, C4H4N2O3 (Merck, Germany)] was added to the supernatant and placed in a boiling Bain-marie for 30 minutes. In the next step, its absorbance was read at 532 nm using a spectrophotometer (UV-3600 Plus, Shimadzu, Japan). To draw the standard MDA curve, different concentrations (mM) of 1, 1, 3, 3-Tetramethoxypropane propane (code: 805797, linear formula: C11H24O4, Merck, Germany) were prepared (24). The MDA concentration was expressed in nmol/mg of protein.

Determination of PC: The PC content was measured by separating excess carbonyl compounds using 2,4-Dinitrophenylhydrazine (DNPH) (Merck, Germany). In summary, some tissue homogenate (0.5 mg protein/ml) was added to an equal volume (0.5 ml) of 0.1% DNPH and incubated at room temperature for 1 hour. This reaction was terminated by the addition of one milliliter of 20% Tricyclic antidepressants (TCA) and the sample protein precipitated. The protein was concentrated by centrifugation at 10,000 rpm and the supernatant discarded. The additional uncomined DNPH was extracted three times using the ethyl acetate/ethanol solution (1:1) (Molecular formula: C4H8O2, Cas Number: 141-78-6, Merck, Germany). The recycled cellular protein was then dried under a stream of dry nitrogen and dissolved in 1 ml of guanidine Tris-HCl buffer solution (8 M, pH = 7.2). The resulting hydrazone solution was measured at 370 nm and the PC concentration was expressed in nmol/mg of protein (25).

Determination of the SOD and GPX gene expression level: The heart tissues were stored at -80 °C until complete RNA isolation. The total RNA was isolated from the heart tissue using RNX Plus™ solution (kit code: EX6101, CinnaGen Company, Iran,) according to the manufacturer's instructions. The concentration of the isolated RNA was determined by determining the absorbance at 260 nm using a NanoDrop device (NanoDrop Technologies, Wilmington, DE, Fisher Scientific, USA). Then, the cDNA synthesis (cDNA synthesis Kit, lot: 201905, cat: yt4500, Yekta Tajhiz Azma Company, Tehran, Iran) was performed according to the method of Han et al. (26).

The relative expression of the SOD and GPX genes was measured using real-time polymerase chain reaction (RE-time PCR) and 5.5 µl SYBR® Green Master Mix (Fermentas Life Sciences, St Leon-Rot, Germany) according to the manufacturer's instructions. All RE-time PCR reactions were performed on a Rotor GeneTM 6000 (Corbett) device. The time-heating schedule of the device was set in three steps. The first stage, which led to the denaturation of cDNA molecules, was 95 °C for 5 minutes, the second stage was 95 °C for 10 seconds for denaturation, 65 °C for 10 seconds for annealing, and 60 °C for 24 seconds for extension in 40

consecutive cycles, and in the final stage for drawing the melting curve, the temperature was increased from 50 to 99 °C in each repetition, 1 degree every 5 seconds.

The relative standard method was applied to calculate the DNA expression level. The melting curve for the SOD and GPX genes (as the internal control genes) marked a peak at the melting point, indicating a specific PCR response for all genes studied (27). Electrophoresis of these products also confirmed this (Figure 2).

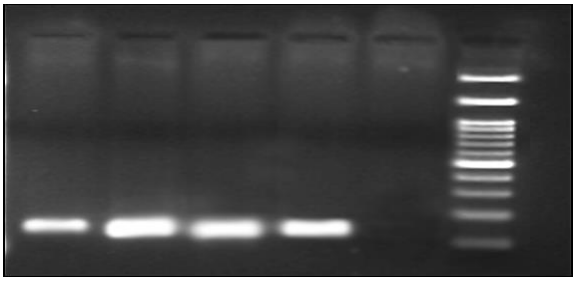

Figure 2. Investigation of the polymerase chain reaction (PCR) product related to amplification of related genes using 2% agarose gel electrophoresis

The quantitative analysis of the data was performed using Equation 1.

Equation 1: Gene expression analysis

$$R = E_{\text{Gene target}}^{\Delta Ct\ \text{Gene target}} / E_{\text{Internal control gene}}^{\Delta ct\ \text{Internal control gene}}$$

In this regard, first the standard curve, efficiency, and the ΔCT difference of the reference genes to target genes were calculated and then the increase or decrease of gene expression in the treatment groups was calculated compared to the control group (28).

The primers used in the present study are presented in table 1.

The details of the present study were approved by the ethics committee of Gerash University of Medical Sciences, Fars Province, Gerash, Iran with the ethics code IR.GERUMS.REC.1398.1085. Moreover, the study was conducted in compliance with ethics standards. The animals were kept based on the standard conditions of care for rats and the animals were sacrificed ethically (29).

The data were analyzed in Prism software (Vesrion 5, Graphpad, Canada). All statistical comparisons were performed using one-way analysis of variance (ANOVA) and Tukey post hoc test. $P \leq 0.05$ was considered as the data significance level.

Table 1. Primers used in the present study

Primer probe	Primer set	Amount generated
SOD	F: GAATTGCCGATGTACGTCG R: GTAGAAAGTGGGGAGGAT	bp
GPX	F: GTGTACCAGTCCGGGTAT R: CCAAAATGCGTTAAACCG	bp

SOD: Superoxide dismutase; GPX: Glutathione peroxidase

Results

The mean weight of the rats was 220 ± 20 g. The results of the level of oxidative stress indices (MDA, PC) and expression of the SOD and GPX genes to evaluate the effects of HIIT and NMN supplementation in the heart tissue of the rats are presented in table 2.

Results of assessment of MDA in heart tissue: In relation to MDA, the control and sham groups had a slight increase compared to the pre-test control group, but in general there was no significant difference between the pre-test control group and the control and sham groups ($P > 0.050$). Decreased MDA was observed in the HIIT, NMN, and NMN + HIIT groups compared to the pretest control, control, and sham groups. This decrease was significant in the NMN and NMN + HIIT groups ($P < 0.001$), with the highest decrease in MDA reported in the NMN + HIIT group ($P < 0.001$) (Table 3).

Results of PC assessment in the heart tissue: PC in different groups showed a slight increase in the control and sham groups compared to the pre-test group, but this increase was not significant and no significant difference was observed between the pre-test control group and the control and sham groups ($P > 0.050$).

Table 2. Mean levels of oxidative stress-antioxidant indices in the study groups

Groups	MDA (nmoles/mg)	PC (nmoles/mg)	SOD gene expression	GPX gene expression
Pre-test control	35.06 ± 3.17	38.36 ± 4.09	5.92 ± 1.18	7.38 ± 1.91
Control	37.55 ± 4.49	42.95 ± 5.93	5.55 ± 1.62	6.31 ± 1.04
Sham	38.12 ± 2.38	39.97 ± 6.10	5.08 ± 1.14	7.20 ± 1.20
HIIT	33.89 ± 3.40	32.71 ± 5.28	7.68 ± 2.24	9.50 ± 1.56
NMN	29.02 ± 5.25	34.25 ± 3.06	8.19 ± 1.91	8.92 ± 1.66
NMN + HIIT	21.09 ± 3.06	26.09 ± 4.70	8.55 ± 1.02	10.32 ± 1.57
Pre-test control	35.06 ± 1.07	38.36 ± 4.09	5.92 ± 1.18	7.38 ± 1.91

Data are reported as mean ± standard deviation (SD).
MDA: Malondialdehyde; PC: Protein carbonyl; SOD: Superoxide dismutase; GPX: Glutathione peroxidase; HIIT: High intensity interval training; NMN: Nicotinamide mononucleotide

The amount of PC in the HIIT group compared to the control group, in the NMN + HIIT group compared to the pretest control and control groups, and in the NMN + HIIT group compared to the pretest control, control, and sham groups showed a significant decrease ($P < 0.050$), with the highest decrease in PC reported in the NMN + HIIT group (Table 4).

Results of SOD gene expression in the heart tissue: The real-time PCR results indicated that the SOD gene expression decreased in the control and sham groups compared to the pre-test control group, but this decrease was not significant ($P > 0.050$). In the HIIT, NMN, and NMN + HIIT groups, the SOD gene expression increased compared to the pretest control and control groups, which was not significant ($P > 0.050$). Furthermore, there was no significant difference between the three treatment groups of HIIT, NMN, and NMN + HIIT ($P > 0.050$) (Table 5).

Results of GPX gene expression in heart tissue: The results in this field revealed that despite the decrease in the GPX level in the control and sham groups compared to the pretest control group, this decrease was not significant ($P > 0.050$). In the HIIT and NMN + HIIT groups, the GPX gene expression was significantly increased compared to the pretest control, control, and sham groups ($P < 0.050$), with the highest increase in the GPX gene expression observed in the NMN + HIIT group ($P <0.050$) (Table 6).

Discussion

Aging is accompanied by an increase in free radicals, which leads to oxidative stress and damage to macromolecules (30). Oxidative stress is a condition in which the balance between the production of free radicals and the level of antioxidants is significantly disrupted. These radicals attack cellular macromolecules, including lipids, proteins, and nucleic acids located in the cell membrane, cytosol, and nucleus, and ultimately lead to cell damage (31).

The body of mammalians uses different types of antioxidants to fight free radicals, maintain a balance between oxidants and antioxidants, and prevent oxidative stress. Antioxidants such as SOD, GPX, and catalase are the body's first line of defense against oxidative damage (32,33). CVDs have a heterogeneous pathophysiological mechanism. Research has shown that many CVDs are associated with overproduction of ROS. Therefore, increased oxidative stress is considered as a potential cause (34).

Under normal circumstances, cardiac aerobic metabolism is associated with the continuous production of peroxidants such as ROS, which are inactivated by the cardiac antioxidant system. Under oxidative stress, overproduction of ROS causes lipid peroxidation and MDA production, resulting in damage to heart tissue (35).

Aerobic organisms are equipped with integrated antioxidant systems, including enzymatic and non-enzymatic systems. The main enzymatic antioxidants in the heart are SOD, GPX, and catalase. Non-enzymatic antioxidants include vitamins (including C and E), beta-carotene, uric acid, and glutathione (GSH) (5). With age, the reduced efficiency of the antioxidant system and its inability to neutralize free radicals trigger mitochondrial and nuclear DNA mutations and increase the risk of CVD (36). An increase in some biomarkers such as MDA and a decrease in antioxidant compounds and antioxidant enzymes indicate the presence of oxidative stress.

In the present study, to confirm the effect of HIIT and NMN supplementation on reducing oxidative stress, some indicators related to oxidative stress including SOD and GPX gene expression, as well as MDA and PC levels in cardiac tissue were measured, with the results suggesting that aging in control animals was associated with an increase in both MDA and PC and a decrease in the expression of the SOD and GPX genes. Administration of the NMN supplementation and HIIT resulted in significant changes in the antioxidant status of the heart tissue. HIIT training and NMN supplementation in each of the independent intervention groups increased the SOD and GPX levels and decreased MDA and PC in the heart tissue of the rats. The highest increase in the SOD and GPX gene expression and the highest decrease in the MDA and PC gene expression were observed in the combined group that underwent the HIIT intervention and the NMN supplementation simultaneously.

The NMN supplementation and HIIT increased the SOD and GPX gene expression. This finding suggests that the reduction in the oxidative stress may be due to the increased antioxidant defense mechanisms, which was consistent with the results of the study by Hellsten et al. They concluded that eight weeks of exercise resulted in a significant increase in skeletal muscle antioxidant enzymes (36).

In addition, the findings support the hypothesis that oxidative stress plays an important role in age-related cardiac dysfunction and suggest that reduction of oxidative stress may be the main mechanism by which NMN and HIIT apply their beneficial effects in older animals. These observations were consistent with the results of the study by de Picciotto et al. (18). In their experiment, the C57Bl/6 rats received NMN supplementation (at a dose of 300 mg/kg/day) in drinking water for 8 weeks.

Table 3. Confidence interval and significance comparison of groups in one-way analysis of variance (ANOVA) test for the malondialdehyde (MDA) index

MDA (nmoles/mg)	Groups	Pre-test control	Control	Sham	HIIT	NMN	NMN + HIIT
Mean ± SD		38.36 ± 4.09	42.95 ± 5.93	39.97 ± 6.10	32.71 ± 5.38	34.25 ± 3.06	26.09 ± 4.70
95% confidence interval of	Pretest control						
intergroup differences	Control	-3.122-8.092					
	Sham	-2.552 -8.662	-5.122-6.177				
	HIIT	-4.432-6.782	-1.947-9.267	-1.377-9.837			
	NMN	-0.437-11.650*	-2.923-14.140*	-3.493-14.710*	-0.737-10.480*		
	NMN + HIIT	8.366-19.580*	10.850-22.070**	11.420-22.640*	78.171-18.410*	2.231-13.540*	
P (Intergroup difference)	Pretest control						
	Control	> 0.999*					
	Sham	0.688	> 0.999*				
	HIIT	0.225	0.506	> 0.999*			
	NMN	0.337*	0.015*	0.020*	> 0.999*		
	NMN + HIIT	≤ 0.001*	≤ 0.001*	≤ 0.001*	≤ 0.001*	≤ 0.001*	

*P < 0.050, **P < 0.050

SD: Standard deviation; MDA: Malondialdehyde; HIIT: High intensity interval training; NMN: Nicotinamide mononucleotide

Table 4. Confidence interval and significance comparison of groups in one-way analysis of variance (ANOVA) test for Protein carbonyl (PC) index

PC (nmoles/mg)	Groups	Pre-test control	Control	Sham	HIIT	NMN	NMN + HIIT
Mean ± SD		38.36 ± 4.09	42.95 ± 5.93	39.97 ± 6.10	32.71 ± 5.38	34.25 ± 3.06	26.09 ± 4.70
95% confidence interval of	Pretest control						
intergroup differences	Control	-12.660-3.210					
	Sham	-10.350 -7.124	-5.764-11.710				
	HIIT	-3.089-14.390	19.925-4.055*	-1.747-16.000			
	NMN	18.890-3.020*	-23.615-7.745*	3.014-14.460	-7.199-10.280*		
	NMN + HIIT	13.525-21.000*	8.115-25.590**	11.420-22.640*	5.140-22.620	-2.125-15.350	
P (Intergroup difference)	Pretest control						
	Control	> 0.999*					
	Sham	0.948	0.340				
	HIIT	0.101	≤ 0.001*	> 0.999*			
	NMN	0.002*	≤ 0.001*	0.322	> 0.999*		
	NMN + HIIT	≤ 0.001*	≤ 0.001*	0.015*	0.195	> 0.999*	

P < 0.050*

SD: Standard deviation; PC: Protein carbonyl; HIIT: High intensity interval training; NMN: Nicotinamide mononucleotide

Table 5. Confidence interval and significance comparison of groups in one-way analysis of variance (ANOVA) test for superoxide dismutase (SOD) index

SOD (nmoles/mg)	Groups	Pre-test control	Control	Sham	HIIT	NMN	NMN + HIIT
Mean ± SD		5.92 ± 1.18	5.55 ± 1.62	5.08 ± 1.14	7.86 ± 2.24	8.19 ± 1.91	8.55 ± 1.02
95% confidence interval of intergroup differences	Pretest control						
	Control	-3.222-3.952					
	Sham	-2.743-4.430	-3.108-4.065				
	HIIT	-5.512-1.662	-5.877-1.297	-6.355-0.818			
	NMN	-5.853-1.320	-6.218-0.955	-6.697-0.476	-3.928-3.245		
	NMN + HIIT	-5.377-1.797	-5.742-1.432	-6.220-0.953	-3.452-4.063		
P (Intergroup difference)	Pretest control						
	Control	> 0.999					
	Sham	0.429	0.766				
	HIIT	0.359	0.649	> 0.999			
	NMN	0.181	0.339	> 0.999	> 0.999		
	NMN + HIIT	0.466	0.828	> 0.999	> 0.999	> 0.999	

SD: Standard deviation; SOD: Superoxide dismutase; HIIT: High intensity interval training; NMN: Nicotinamide mononucleotide

Table 6. Confidence interval and significance comparison of groups in one-way analysis of variance (ANOVA) test for Glutathione peroxidase (GPX) index

GPX (nmoles/mg)	Groups	Pre-test control	Control	Sham	HIIT	NMN	NMN + HIIT
Mean ± SD		7.38 ± 1.91	6.31 ± 1.04	7.20 ± 1.20	9.50 ± 1.56	8.92 ± 1.66	10.32 ± 1.57
95% confidence interval of intergroup differences	Pretest control						
	Control	-1.602-3.752					
	Sham	-2.497-2.857	-3.572-1.782				
	HIIT	-4.795-0.558	-5.870-0.516	-4.975-0.378			
	NMN	-4.214-1.140	-5.289-0.065	-4.394-0.960	-2.095-3.259		
	NMN + HIIT	-5.615-0.261	-5.795-0.440	-3.497-1.857	-4.097-1.275		
P (Intergroup difference)	Pretest control						
	Control	> 0.999					
	Sham	> 0.999	> 0.999				
	HIIT	0.158	0.017*	0.348			
	NMN	0.706	0.092	> 0.999	> 0.999		
	NMN + HIIT	0.015*	0.001*	*0.036	> 0.999	> 0.999	

*P < 0.05

SD: Standard deviation; GPX: Glutathione peroxidase; HIIT: High intensity interval training; NMN: Nicotinamide mononucleotide

de Picciotto et al. concluded that NMN supplementation restored the SIRT1 activity and reversed age-related vascular function by reducing oxidative stress. These effects were associated with improved NO bioavailability, reduction of oxidative stress, and normalization of structural proteins in vascular walls (18).

In another study, Bogdanis et al. examined the short-term effects of HIIT on oxidative stress and antioxidant status and found that three weeks of HIIT reduced oxidative stress markers and increased antioxidant levels (7). HIIT seems to be a new method of long-term endurance training, with adaptations to increase the gene expression of antioxidant enzymes, improve cell metabolism, and increase cell longevity (37).

The findings of several studies have indicated that aging reduces NAD^+ and predisposes the body to a wide range of chronic diseases and pathological conditions associated with aging (17,38). There is strong evidence that restoring cellular NAD^+ levels in older animals using NAD^+ precursors has potent anti-aging effects and reverses age-related complications (17). In a study, Kiss et al. concluded that age-related reductions in NAD^+ could be compensated for by NMN supplements as NAD^+ precursors. They found that age-related increases in H_2O_2 production were reduced by NMN supplementation, in addition, it can reduce age-related mitochondrial oxidative stress (17). Furthermore, the results of recent studies showed that the use of NMN supplementation increases SIRT1 and NAD^+ and decreases oxidative stress in heart tissue (17).

Limitations

Given the type of the study, control of anxiety and stress in animals during exercise, which may affect the variables of the present study, was not completely possible. Due to the complexity of the antioxidant system-oxidative stress, it seems that the lack of measurement of DNA damage indices and total antioxidant capacity (TAC) is one of the limitations of this study. Therefore, it is suggested that more oxidative stress indices be measured in future studies.

Recommendations

In order to complete the present study, it is suggested that in future studies, the effects of these two interventions be investigated on other tissues of the rat body, such as muscle tissue, brain, and liver. The results of the present study showed that HIIT training and NMN supplementation increase the SOD and GPX levels and decrease MDA and PC levels in rat heart tissue. Therefore, it is suggested that the HIIT training and a supplement similar to that of the present study be used to improve antioxidant function in heart tissue. It is also recommended that the same study be performed on rats older than 2 years.

Conclusion

The results of the present study revealed that administration of NMN and HIIT supplements for 8 weeks reduced age-related oxidative stress in cardiac tissue. Besides, co-administration of the NMN supplement and HIIT had the greatest effect on reducing oxidative stress. Accordingly, it seems that performing HIIT and NMN supplementation separately and in different ways, leads to improving the status of antioxidants in heart tissue. Overall, the NMN supplementation with antioxidant properties, and HIIT by establishing a peroxidant-antioxidant balance reduced age-related oxidative stress in the heart tissue.

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Authors' Contribution

Sakineh Taheri: study design and ideation, attracting financial resources for the study, study support, executive, and scientific services, providing study equipment and samples, data collection, 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 to maintain the integrity of the study process from the beginning to the publication, and responding to the referees' comments; Sajad Arshadi: study design and ideation, study support, executive, and scientific services, analysis and interpretation of results, specialized statistics services, 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 to maintain the integrity of the study process from the beginning to the publication, and responding to the referees' comments; Abdolali Banaeifar: study support, executive, and scientific services, analysis and interpretation of results, specialized statistics services, 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 to maintain the integrity of the study process from the beginning to the publication, and responding to the

referees' comments; Vahid Imanipour: study support, executive, and scientific services, analysis and interpretation of results, specialized statistics services, 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 to maintain the integrity of the study process from the beginning to the publication, and responding to the referees' comments.

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Conflict of Interest

The authors declare no conflict of interest. Dr. Sajad Arshadi has been working as a faculty member since 2011 and as an assistant professor at the South Tehran Branch, Islamic Azad University since 2014. Dr. Abdolali Banaeifar has been working as a faculty member since 2002 and as an associate professor at the South Tehran Branch, Islamic Azad University since 2016. Dr. Vahid Imanipour has been working as a faculty member since 2005 and as an assistant professor at Parand Branch, Islamic Azad University since 2017. Sakineh Taheri has been studying as a PhD student in sports physiology at the Faculty of Physical Education and Sports Sciences, South Tehran Branch, Islamic Azad University since 2016.

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