Iran J Public Health, Vol. 44, No.9, Sep 2015, pp. 1270-1276

Original Article

Cardioprotective Effect of High Intensity Interval Training and Nitric Oxide Metabolites (NO_2^-, NO_3^-)

Aliasghar FALLAHI [1, 2], Abbasali GAEINI [3], Shahnaz SHEKARFROUSH [4], Ali KHOSHBATEN [2]

1. *Dept. of Physical Education and Sport Science, School of Education and Psychology, Shiraz University, Shiraz, Iran*
2. *Sport Physiology Research Center, Baghiyatallah University of Medical Sciences, Tehran, Iran*
3. *Dept. Exercise Physiology, Faculty of Physical Education and Sport Science, Tehran University, Tehran, Iran*
4. *Dept. of Physiology, Arsanjan Branch, Islamic Azad University, Fars, Iran*

***Corresponding Author:** Email: alifallahi@ut.ac.ir.

(Received 14 Dec 2014; accepted 25 May 2015)

Abstract

Background: The aim of this study was to investigate the effects of High-Intensity Interval Training (HIIT) on nitric oxide metabolites ($NO2^-$, $NO3^-$) and myocardial infarct size after Ischemia/Reperfusion (I/R) injury in healthy male rats.

Methods: A total of 44 Wistar rats were randomly divided into 4 groups including HIIT (n=8), HIIT + IR protocol (n=14), control (n=8), and control + IR (n=14). Each training session of HIIT consisted of 1 hour of exercise in three stages: 6-minute running at 50-60% VO2max for warm-up; 7 intervals of 7-minute running on treadmill with a slope of 5^0 to 20° (4 minutes with an intensity of 80-100% VO2max and 3 minutes at 50-60% VO2max); and 5-minute running at 50-60% VO2max for cool-down. The control group did not participate in any exercise program. Nitric Oxide (NO) and its metabolites were measured by using Griess reaction test.

Results: The results showed that eight weeks of exercise training exerted a significantly increasing effect on nitrite (8.55 µmol per liter, equivalent to 34.79%), nitrate (62.02 µmol per liter, equivalent to 149.48%), and NOx (66 µmol per liter, equivalent to 98.11%) in the HIIT group compared with the control group. The results showed myocardial infract size (IS) was significantly smaller (23.2%, $P<0.001$) in the exercise training group compared with the control group.

Conclusion: Incremental changes in NO-NO_3^-, NO_2^- axis are one of mechanisms through which HIIT program can protect the heart from I/R injury and decrease myocardial infarction.

Keywords: High-intensity interval training, Cardioprotection, Preconditioning, NO, NO_2^-, NO_3^-

Introduction

Cardiovascular diseases are still the main cause of mortality all over of the world. Therefore, cardioprotection is one of the most important criteria in recent decades. In patients who survive acute myocardial infarction (AMI), the amount of damaged myocardium due to ischemia (infract size) largely predicts the mortality rate of patients. Thus, it is commonly held that a significant decline in myocardial infarction decreases the morbidity and mortality. Preconditioning (PC) is one of the main approaches to decrease myocardial infarction (1). Ischemic preconditioning (IPC) refers to a brief period of ischemia protecting the heart against prolonged episodes of ischemia (2). Despite the considerable protective effects of IPC, it is not applicable in the clinic. Regular exercise training is one of the most efficient PC methods.

Exercise protects myocardium against ischemia reperfusion (IR) injury (3). However, the mechanism of these protective functions is not fully

known to date. Several mechanisms have been examined concerning the cardioprotective effects of exercise training. NO makes a greater contribution to cardiovascular health than was expected before. NO is both a trigger (through endothelial NOS) and a mediator (through iNOS) of delayed ischemic preconditioning (4). Moreover, NO has several physiological properties that highlights it as the cardioprotective signaling molecule that is potentially involved in myocardial I/R injury (5-8). ENOS-deficient rats ran a distance 60% less than that of their same-age counterparts in the control group (8). Considering the cardioprotective effects of NO and its instability, some researchers have conducted more studies on the NO effects and its metabolites, nitrite and nitrate. Nitric oxide amounts decrease in endothelial dysfunction (9). Exercise training, especially high intensity interval training (HIIT) may restore NO concentration and its metabolites to a normal range and decrease vascular disorders (10). NO concentration decreases during ischemia and reversely the plasma levels of the NO metabolites increase during exercise in both rodents and human (11, 12).

Increased levels of NO metabolites during exercise may contribute to cardiac protection against IR injury. Since there is limited evidence on the role of nitrite and nitrate in exercise-induced cardioprotection, the present study aimed to address the variations in the plasma levels of nitric oxide metabolites following eight weeks of HIIT and its cardioprotective effects during IR injury in adult male rats.

Materials and Methods

Research samples

A number of 44 male Wistar rats, 3 - 4 months, were prepared from Iranian Pasteur Research Center and used in the present study. The rats were kept in the 12:12 light–dark cycle at 50% moisture and a temperature of 22 ± 3^{0} C. They were kept in cages containing 4 rats per unit and had free access to food.

The ethical codes of treating laboratory animals, set by the Iranian Society for Supporting Laboratory Animals Used for Scientific Purposes, were strictly followed in the present study. The University of Tehran approved the treating and handling method, the training program and the sampling type of animals conducted in the present study.

Allowing the animals to adapt to their new environment for a week, they were divided into 4 groups including HIIT (n=8), HIIT+IR program (n=14), control (n=8), and control+IR program (n=14).

Exercise Training program

The training program was developed based on previous protocols (13, 14) and the researchers' experiences. During the first week, the animals were introduced into the training program by walking and then running on the treadmill at different speeds. Then, they performed 8 weeks of progressive exercise training. Every session consisted of one hour exercise training performed in three stages:

- Warm-up: running for 6 minutes at 50-60% VO2max
- Main training: 7 intervals of 7-minute running at $5\text{-}20^{0}$ slope consisting of 4-minute running at 80-100% VO2max and 3-minute running at 50-60% VO2max
- Cool-down: running for 5 minutes at 50-60% VO2max

The control group did not perform any exercise training whatsoever. However, in order to create similar conditions for all the subjects, the control rats were placed on the immobile treadmill 3-5 times a week for 10-15 minutes per session in order to adapt to the environment (15). Since the best time for rats is during the dark cycle to do exercise training (16), the training program was conducted in the afternoon during the dark cycle (between 6 p.m. and 12 a.m.) under red light (16).

Assessment of the aerobic power of rats

In order to examine the aerobic power of rats, the indirect protocol was used with high accuracy based on previous experiments (14, 17, 18). The intensity of the training program in terms of Vo2max was obtained based on the relation of Vo2max to speed and treadmill slope.

I/R protocol and measuring myocardial infarction size (IS)

The rats were anesthetized by intraperitoneal injection of ketamine (50 mg/kg body weight) and pentobarbital sodium (30 mg/kg body weight). The rectal temperature of animals was maintained at 37±0.5 ^{0}C over the course of study. Following the shaving of hair on the neck and the left side of the chest, the animals were fixed on the autopsy table containing a thermal pad. A standard limb lead II electrocardiogram was monitored and recorded throughout the experiment.

Subsequently, the neck was cut in the midline and the muscles were pushed apart. Then, the trachea was cannulated and attached to a ventilator. The ventilator was adjusted at 80 breaths per minute and a volume of 1 ml per 100 grams of body weight. Subsequently, the left carotid artery was cannulated and attached to the pressure transducer in order to record the blood pressure. The thorax was opened from the fourth and fifth intercostal space and the pericardium was torn to pass the 6-0 silk suture under the LAD artery (between the tip of the left atrium and pulmonary artery). Allowing 10-15 minutes for the animal to return to stable conditions, we started the ischemia stage. When the animal regained its stable conditions, either end of the suture passed from under the LAD artery was passed and pulled into a tube (part of the sampler head) and fixed with another tube. The LAD artery blockage continued for 30 minutes, which was considered as the ischemia stage (19). Following the blockage of LAD artery, a number of symptoms usually occur, such as immediate drop in blood pressure, faded heart tip and ST elevation in the ECG, which are the main symptoms of artery blockage. In this stage, it is expected that arrhythmia occurs about 4-7 minutes after the artery blockage and continues for about 10-15 minutes. Following the ischemia stage, the artery was opened and reperfusion resumed for 90 minutes (17). At the end of reperfusion stage, the LAD artery was blocked again. In the rats used to collect samples, the cardiac blood sampling was immediately conducted after this stage and the cardiac tissue was cut off for further experiments. In the rats considered as the samples to determine the infraction size, one ml of evans blue 2% was injected into the right atrium with a syringe in order to distinguish the ischemic from non-ischemic area. Eventually, the heart was immediately cut out, removed by thoracotomy, and was washed with normal saline solution. The atrium and large arteries were cut off, and the heart was put into a cutting matrix and maintained in -20 ^{0}C (17). After freezing, a number of five 2-mm cuts were made from the tip to the base of the heart. The cuts were then put on a lid Canon 25 scanner and carefully scanned from either side with an accuracy of 300 dpi. Subsequently, the cuts were incubated in a solution containing TTC 1% at 37 ^{0}C for 20-30 minutes in order to distinguish the infracted (white) from non-infracted (red) areas. In order to distinguish the red and white areas further, the cuts were fixed in formalin solution 10% for about 24 hours. They were then inserted on lid Canon 25 scanner and scanned on either side with an accuracy of 300 dpi. The ischemic and infracted areas on either side of the cuts were measured using Toll Image software and averages were obtained. Finally, the infraction size was reported as a percentage of ischemic area.

Sampling procedure

In order to measure the biochemical parameters, 8-10 ml whole blood was taken from every rat heart and discharged into tubes lacking any anticoagulant or other additives. The samples were immediately centrifuged at 3500 rpm for 15 minutes. The obtained plasma was poured into 4 microtube vials and preserved at -70 ^{0}C for later measurement.

Measurement of NO metabolites

In order to measure serum NOx, we used griess reaction method as reported in previous researches (6, 20).

Statistical Analysis

All values are expressed as mean ± SEM. Independent t-test was used to assess the difference between the two groups for each of the variables. $P \leq 0.05$ was considered to reflect a significant difference between control and HIIT values.

Analysis was performed using SPSS for Windows, version 16 (Chicago, IL, USA).

Results

Body and muscle mass ratio of groups is shown in Table 1 and 2.

The exercise-training program exerted an incremental, significant effect on nitrite, nitrate and NOx concentration. Compared with the control group, the intervention increased the amounts of nitrite by 34.79% (8.55 µmol/L), nitrate by 149.48% (62.02 µmol/L), and NOx by 98.11% (66 µmol/L) in the training subjects. In addition, there were significant differences in the myocardial infract size (IS) between the two groups. IS was smaller in the HIIT group as much as 23.02% compared with the control group (P<0.001) (Table 3).

Table 1: Body mass of groups before and after program

Body Mass (g)	**Pre**		***P*-value**	**Post**		***P*-value**
	Control	**HIIT**		**Control**	**HIIT**	
	257.37±13.56	247.12±14.69	0.169	330.50±21.36	304.37±19.44	0.023*
P-value pre-post Control			P <0.001*			
P-value pre-post HIIT				P <0.001*		

Data are shown as Mean ± SEM

Table 2: Muscle mass including ratio of heart, extensor digitrum longous (EDL), gastrocinemus (Gast), tibia and soleous (Sol) to body mass in HIIT and control groups

Muscle	**HIIT**	**Control**	***P*-value**
Heart mass (mg/g)	1.10 ± 0.04	0.96± 0.06	0.001
Heart mass/body mass	3.32±0.95	2.91± 0.071	0.001
EDL/body mass	0.67± 0.15	0.53± 0.06	0.069
Gast/body mass	7.08± 0.27	6.75±0.68	0.255
Tibia /body mass	2.23±0.06	2.01±0.15	0.003
Sol/body mass	0.53± 0.08	0.50± 0.64	0.380

Data are shown as Mean ± SEM.

Table 3: The amounts of nitrite, nitrate, Nox and IS percent (the ratio of IS to AAR) (Mean±SD) in HIIT and control rats following 8 weeks of exercise training

Variable	**HIIT**	**Control**	***P* value**
Nitrite (µmol/L)	33.12± 2.51	24.57± 1.24	0.009
Nitrate(µmol/L)	103.51± 8.72	41.49± 9.21	< 0.001
NOX (µmol/L)	133.27± 9.93	67.27± 10.38	< 0.001
Myocardial infract size (IS) (%)	19.26±9.59	42.28±11.74	< 0.001

Data are shown as Mean ± SEM

Discussion

This is one of the first investigations on the cardioprotective effects of HIIT by measuring the indices of NO-NO_2^-, NO_3^- axis. The main finding of the present study was that incremental changes in serum NO and its metabolites (nitrite and nitrate) following an HIIT program may decrease the myocardial infract size and protect myocardium after ischemia/reperfusion injury. Therefore, NO-NO_2^-- NO_3^-axis can be one of the exercise-induced cardioprotective mechanisms. Although some studies have already addressed this hypothesis, it has only been investigated experimentally by

Calvert and colleagues who developed a 4-week voluntary training program (21). Accordingly, they found that 4 weeks of voluntary training on the running wheel might contribute to cardioprotection by stimulating Beta-3 adrenergic receptor and increasing NO metabolites supply including nitrite and S-Nitrosothiols.

Increased content of NO synthase (NOS) is not necessary for the short-term protection of myocardium following exercise training. These findings suggest that there is a significant difference between exercise-induced cardioprotection and other types of delayed preconditioning including ischemic preconditioning (22). The effect of 6 months of exercise training has been investigated on hematic nitrite and nitrate concentration in postmenopausal women suffering hypertension (23). Their training program consisted of 20-60 minutes of continuous exercise training on ergometer bicycle at 50% heart rate reserve. Significant decreases in systolic and diastolic blood pressure were associated with a considerable increase in NO concentration (10±0.9 μM to 16±2 μM).

Gomes and colleagues reported that three months of exercise training on the stationary bicycle at the intensity of 10% below the individual anaerobic threshold increased NO bioactivity and NO indicators (whole blood nitrite and CGMP) and decreased oxidative stress in the patients with Metabolic Syndrome. They found that these biochemical effects were associated with decreased hematic levels of endogenous NO synthase inhibitors, such as asymmetrical dimethylarginine (ADMA) (24).

In the present study, the results revealed that HIIT program significantly decreased myocardial infract size in the training group compared with the control group by increasing blood and, perhaps, cardiac NO supply (98.11% increase in NOX). Endothelium plays an important role in the cardioprotective effects of exercise training. Specifically, increased exercise-induced shear stress of the vascular wall increases eNOS expression and activity and thus increases NO bioavailability and production across the body (25).

Inhibition of endogenous NO by using NO synthase inhibitors, such as NG-nitro-L-arginine methyl ester (L-NAME) and NG-monomethyl-L-arginine (L-NMMA), increases blood pressure and vasoconstriction as well as the movement, turbulence and adhesion of leukocytes (19). Although the present study did not aim to examine blood pressure variations, previous findings suggest that another aspect of cardioprotective mechanism is associated with increased nitrite and nitrate concentrations in response to general and local blood pressure in a part of the heart that has suffered ischemia/reperfusion injury. This, in turn, would decrease the amount of tissue needs for oxygen so that cellular death and degeneration decrease. Research has shown that hematic concentration of nitrite and nitrate affects their tissue supply. The main sources of nitrite and nitrate are oxidative breakdown of NO produced by NOS isoforms (mostly eNOS), nutrients and foods containing nitrite and nitrate. However, it is not clearly known how much of nitrite and nitrate is supplied by the shear effect and eNOS enzyme and how much of them is provided by food and nutrients. Besides, since fresh water also contains nitrite and nitrate, the amount of water intake, absorption and discharge can affect the hematic and tissue concentration of nitrite and nitrate. As the nitrite and nitrate concentrations were significantly higher in the training groups compared with the control group, the amount of produced NO, as well as the nitrite and nitrate produced due to the shear stress of intermittent increase in the blood flow following HIIT, must have been large enough to account for a part of cardioprotective effect of exercise training by decreasing the myocardial infract size.

Conclusion

Incremental changes in NO^-, NO_3^-, NO_2^- axis may be one of the mechanisms through which an HIIT program can contribute to myocardial protection and decrease myocardial infract size after ischemia/reperfusion injury.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or fal-

sification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgement

We thereby thank Dr. Mohammadi, Dr. Asgari, who contributed to this research. This work was supported by grant from the Sport Physiology Research Center, Baghiyatallah University of Medical Sciences, and Iran National Science Foundation, Tehran, Iran. The authors declare that there is no conflict of interests.

References

1. Calvert JW (2011). Cardioprotective effects of nitrite during exercise. *Cardiovasc Res,* 89(3):499-506.
2. Hanley PJ, Daut J (2005). K(ATP) channels and preconditioning: a re-examination of the role of mitochondrial K (ATP) channels and an overview of alternative mechanisms. *J Mol Cell Cardiol*, 39(1):17-50.
3. 3.Freimann S, Scheinowitz M, Yekutieli D, Feinberg MS, Eldar M, Kessler-Icekson G (2005). Prior exercise training improves the outcome of acute myocardial infarction in the rat. Heart structure, function, and gene expression. *J Am Coll Cardiol*, 45(6):931-8.
4. Heusch G, Boengler K, Schulz R (2008) . Cardioprotection: nitric oxide, protein kinases, and mitochondria. *Circulation*, 4;118(19):1915-9.
5. Thomas DD, Liu X, Kantrow SP, Lancaster JR, Jr (2001). The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O2. *Proc Natl Acad Sci*, 2;98(1):355-60.
6. Yang L, Jia Z, Zhu M, Zhang J, Liu J, Wu P, et al (2014). Exercise protects against chronic beta-adrenergic remodeling of the heart by activation of endothelial nitric oxide synthase. *PLoS One*, 9(5):e96892.
7. Momken I, Lechene P, Ventura-Clapier R, Veksler V (2004). Voluntary physical activity alterations in endothelial nitric oxide synthase knockout mice. *Am J Physiol Heart Circ Physiol*, 287(2):914-20.
8. Ojaimi C, Li W, Kinugawa S, Post H, Csiszar A, Pacher P, et al (2005). Transcriptional basis for exercise limitation in male eNOS-knockout mice with age: heart failure and the fetal phenotype. *Am J Physiol Heart Circ Physiol*, 289(4):1399-407.
9. Khan M, Mohan IK, Kutala VK, Kumbala D, Kuppusamy P (2007). Cardioprotection by sulfaphenazole, a cytochrome p450 inhibitor: mitigation of ischemia-reperfusion injury by scavenging of reactive oxygen species. *J Pharmacol Exp Ther*,323(3):813-21.
10. Heiss C, Lauer T, Dejam A, Kleinbongard P, Hamada S, Rassaf T, et al. (2006). Plasma nitroso compounds are decreased in patients with endothelial dysfunction. *J Am Coll Cardiol*, Feb 7;47(3):573-9.
11. Calvert JW, Elston M, Sindler A, Gundewar S, Grinsfelder DB, Aragon JP, et al. (2010). The generation and storage of nitric oxide metabolites during exercise training contributes to exercise-mediated cardioprotection. *J Am Coll Cardiol*, 55(10):A116. E1081.
12. Calvert JW, Condit ME, Aragón JP, Nicholson CK, Moody BF, Hood RL, et al (2011). Exercise protects against myocardial ischemia-reperfusion injury via stimulation of β(3)-adrenergic receptors and increased nitric oxide signaling: role of nitrite and nitrosothiols. *Circ Res*,108(2):1448-58.
13. Haram PM, Kemi OJ, Lee SJ, Bendheim MO, Al-Share QY, Waldum HL, et al. (2009). Aerobic interval training vs. continuous moderate exercise in the metabolic syndrome of rats artificially selected for low aerobic capacity. *Cardiovasc Res*, 1;81(4):723-32.
14. Hoydal MA, Wisloff U, Kemi OJ, Ellingsen O (2007). Running speed and maximal oxygen uptake in rats and mice: practical implications for exercise training. *Eur J Cardiovasc Prev Rehabil*,14(6):753-60.
15. Mondon CE, Dolkas CB, Sims C, Reaven GM. Spontaneous running activity in male rats: effect of age. *J Appl Physiol*, (1985). 1985 May;58(5):1553-7.
16. Farrell PA, Fedele MJ, Hernandez J, Fluckey JD, Miller JL, 3rd, Lang CH, et al (1999). Hypertrophy of skeletal muscle in diabetic rats in response to chronic resistance exercise. *J Appl Physiol*, 87(3):1075-82.

17. Esposito F, Ronchi R, Milano G, Margonato V, Di Tullio S, Marini M, et al. (2011). Myocardial tolerance to ischemia-reperfusion injury, training intensity and cessation. *Eur J Appl Physiol*, 111(5):859-68.
18. Armstrong RB, Laughlin MH, Rome L, Taylor CR (1983). Metabolism of rats running up and down an incline. *J Appl Physiol Respir Environ Exerc Physiol*, ;55(2):518-21.
19. Jugdutt BI (2002). Nitric oxide and cardioprotection during ischemia-reperfusion. *Heart Fail Rev*, 7(4):391-405.
20. Miranda KM, Espey MG, Wink DA (2001). A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide*, 5(1):62-71.
21. Calvert JW, Condit ME, Aragon JP, Nicholson CK, Moody BF, Hood RL, et al. (2011). Exercise protects against myocardial ischemia-reperfusion injury via stimulation of beta(3)-adrenergic receptors and increased nitric oxide signaling: role of nitrite and nitrosothiols. *Circ Res*, 108(12):1448-58.
22. Taylor RP, Olsen ME, Starnes JW (2007). Improved postischemic function following acute exercise is not mediated by nitric oxide synthase in the rat heart. *Am J Physiol Heart Circ Physiol*, 292(1): 601-607.
23. Zaros PR, Pires CE, Bacci M, Jr., Moraes C, Zanesco A (2009). Effect of 6-months of physical exercise on the nitrate/nitrite levels in hypertensive postmenopausal women. *BMC Womens Health*, 9:17.
24. Gomes VA, Casella-Filho A, Chagas AC, Tanus-Santos JE (2008). Enhanced concentrations of relevant markers of nitric oxide formation after exercise training in patients with metabolic syndrome. *Nitric Oxide*, 19(4):345-50.
25. Kim HK, Thu VT, Heo HJ, Kim N, Han J (2011). Cardiac proteomic responses to ischemia-reperfusion injury and ischemic preconditioning. *Expert Rev Proteomics*, 8(2):241-61.