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ORIGINAL ARTICLE

Effects of Six Weeks of High-Intensity Interval Training on HIF-1α Protein Expression in Lung Tissues and Apoptosis of Pulmonary System in Male Wistar Rats

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ABSTRACT

Background. Apoptosis is a kind of regulated cell death involved in the normal growth, development, immune response, and elimination of abnormal cell proliferation in living organisms. In contrast, regular exercise protects against aging, lipid peroxidation, inflammation, and apoptosis. **Objectives.** This study aimed to investigate the effects of 6 weeks of high-intensity interval training (HIIT) on HIF-1α level in lung tissues and apoptosis of the pulmonary system in male Wistar rats. **Methods.** Ten male Wistar rats were divided into training and control groups for this purpose. They performed six weeks of HIIT conducted in 5 sessions per week, each session for 30 minutes at a speed of 15 to 70 m/min. Then, the animal lung tissue was extracted, and HIF-1α levels and lung tissue apoptosis index were measured via ELISA and immunohistochemical methods, respectively. **Results.** It showed that HIIT significantly increased HIF-1α level p =0.001) and lung and pulmonary duct apoptosis (p=0.001) despite a significant improvement in exercise performance (p=0.001). **Conclusion.** According to the results, HIIT enhances athletic performance, HIF-1α level increases, and the rate of apoptosis rises.

KEYWORDS: ***Exercise Training, Hypoxia, Pulmonary Function, DNA Damage, Neoplasia.***

INTRODUCTION

Apoptosis is essential in maintaining cell number and tissue structure and is an essential mechanism for tissue biocompatibility (1). Failure to initiate apoptosis after the severe DNA damage is a sign of neoplasia (1). Apoptosis is involved in neurological disorders such as Alzheimer's and autoimmune diseases such as Hashimoto's Thyroiditis (2); Apoptosis has also been observed in the cellular epithelium in pulmonary fibrosis (2).

Pre-apoptotic conditions appear to occur during prolonged and severe hypoxia in which HIF-1α (Hypoxia-Induced Factor-1 alpha), along with the p53 gene, plays a determining role (3). Hypoxia-Inducible Factor-1 (HIF-1) is a crucial regulator of molecular responses to hypoxia and mediates a wide range of cellular and physiological mechanisms essential for oxygen uptake and has an essential role in regulating target gene

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expression angiogenesis, blood cell proliferation, energy metabolism, and apoptosis (4). In contrast, it may have detrimental pathophysiological effects of ischemia, diabetes, atherosclerosis, Alzheimer's disease, pulmonary obstruction disease symptoms, inflammatory disorders, and cancer (2).

It has been found that, along with other effects of HIF-1α on body cells, hypoxia and the HIF-1α gene act as a pre-apoptotic or anti-apoptotic factor depending on the cell type and experimental conditions (4). Pre-apoptotic conditions appear to occur during prolonged and severe hypoxia in which HIF-1α and the p53 gene play a critical role (4). Conversely, studies have shown that hypoxia can also exert different effects on other body tissues by expressing the protein of Hypoxia Inducible Factor (HIF). The HIF pathway has recently been indicated to play a vital role in regulating immune and inflammation (5).

It has been reported that moderate-intensity endurance training under standard conditions reduces ciliated epithelial cells, decreases apoptosis, and actively repairs small airways and bronchial epithelium tissue, increasing the number of epithelial cells (6); however, inflammatory activity does not increase (6). These changes may indicate a consistent response to increased ventilation during exercise (6). In contrast, vigorous physical activity is often associated with producing large amounts of oxygen free radicals, which can damage cellular structures such as DNA damage in many organs, including the lung, leading to apoptosis (2).

Lung tissue is essential in athletic activity due to gas exchange at its surface that may be vulnerable to hypoxia-induced by increased intravascular training, which may play a significant role in hypoxia-stimulated HIF-1α protein (7). Extensive and prolonged exercise can cause temporary immune system impairment, increased oxidative stress, or overtraining syndrome for athletes at higher levels (7, 8). This type of exercise can lead to impaired whole-body homeostasis and may serve as a temporary suppressor of the immune system through physical stressors such as hypoxia (9).

Since it has been shown that endurance training reduces apoptosis and improves lung function, researchers currently use appropriate-intensity interval training as a new exercise method to enhance cardiovascular fitness. Thus, the question is whether these training modalities can affect lung tissue apoptosis. In addition, given the hypoxia caused by these exercises, it is hypothesized that HIF will change after training in the lung tissue. Therefore, the present study aimed to investigate the effect of six weeks of high-intensity interval training on apoptosis and HIF-1α levels in the lungs of male Wistar rats.

MATERIALS and METHODS

Animals. Ten male Wistar rats (aged four weeks, mean mass 68 ± 9 gr) were obtained from the Pasteur Institute (Mazandaran, Amol, Iran). After two weeks of familiarizing themselves with the environment and activity on the treadmill, they began training at six weeks of age, and they were divided into two groups exercise and control. Required surveys were carried out. Maintaining conditions in the laboratory where the temperature of 22 ± 1.4 (^{0}C), the humidity of 45-55%, and a light-dark cycle of 12:12 hours in transparent polycarbonate cages with five rats in each cage. During the study, animals were given standard pellet food and water ad libitum.

Study Protocol. Under the training protocol, the familiarizing program consisted of four walking and running sessions at a speed of 10 to 25 m/min and a gradient of zero. The high-intensity interval training program consisted of ten 1-minute repetitions and 2-minute active rest. The low-intensity time rate was half the speed of the run and the total daily exercise time for each subject was 30 minutes. The exercise took place every day except for Thursdays and Fridays. Subjects started the high-intensity interval training program at 25 m/min and completed it at 70 m/min. Apart from the primary activity time, 5 minutes were provided for a warm-up and 5 minutes for a cool down (14). The time to reach exhaustion was characterized by mild shock. When the rats hit the shock device at the end of the treadmill five times within 2 minutes or showed returning reflection and standing upright on their feet, they were considered exhausted (15). The testing protocol consisted of a gradual warm-up with an intensity of 15 to 25 m/min and an endurance performance test with a 65 m/min speed, which was measured and recorded using a timer (16). Forty-eight hours before and after the test, the main exercise program (HIIT) was stopped, and the rats rested. The performance of the groups was calculated according to the following formula (17):

$$\Sigma pri = \Sigma mViTi = \Sigma mDi = mDi$$

Where:

Pri: Performance (Kg.m), m: Weight (Kg), Vi: Speed (m/min), Ti: Time (min), Di: Traveled Distance (m)

Lung Tissue Sampling. Tissue sampling from the lungs of rats was performed at the end of the 6-week study interval training. For this purpose, rats were anesthetized by injecting three units of ketamine (30-50 mg/kg of rats' weight), xylazine (3–5 mg/kg of rats' weight), and their lung tissues were excised. Lung tissues were weighed using a Sartorius: B1 1500 scale, and lung tissue volume was measured using the graduated cylinder and Archimedes Law. The dependent variables included changes in the apoptotic changes of pulmonary epithelial cells. The level of HIF-1α in lung tissue was also analyzed. The right lobes of the lungs of the specimens were fixed in a 10% formalin fixative solution to determine the apoptosis index. For the preparation of microscopic sections, samples were paraffin-embedded. In this method, different passage stages, including dewatering, clarification, and paraffin impregnation, were performed after fixation using the Histokinet Model 2000 manufactured by Lika Company of Germany.

Immunohistochemical Detection of Apoptotic Cells. To detect apoptosis of the lining tissue (bronchi and bronchial), the nuclei of these cells were stained and identified using a non-radioactive in situ end-labeling method. After fixation and standard and usual phases of obtaining tissue sections, three μm thick sections were prepared. The sections were then deparaffinized using two containers of xylene, decanted with the lower alcohol concentrations and washed three times with nuclease-free phosphate buffer. To eliminate endogenous peroxidases, sections were incubated with 0.3% hydrogen peroxide in methanol for 30 min at 15-25 ° C. After washing, the sections were treated with a nuclease-free phosphate buffer with proteinase K. The laboratory kit used in this study was the POD Cell Diagnosis Kit manufactured by the German Rouge Company (Kit 910 817 684 11). All phases were performed per the kit instructions. To determine the apoptosis index at each section, ten high-magnification bronchi and bronchiole were examined, and TUNEL positive (dark brown and uniform nuclei) and negative lining tissue were counted. Then the labeling index (LI) was calculated using the following formula:

$$LI = a/(a+b) \times 100$$

Where "a" is the number of positive TUNEL nuclei and "b" is the number of negative TUNEL nuclei in each region.

Measurement of HIF-1α Levels in Lung Tissue. Another dependent variable included lung HIF-1α levels, which were evaluated and analyzed. Lung HIF-1α levels were determined using the ELISA method (CUSABIO BIOTECH kit_CSB-PA578243_China, with a sensitivity of 0.78 pg/ml). For this purpose, the left lobe of lung tissue was first centrifuged using powdered, liquid nitrogen and then homogenized in buffer solution for 15 minutes at a speed of 3000 g. The obtained solution, using dry ice, was transferred to the laboratory to measure the desired index.

Statistical Analysis. Descriptive statistics were used to measure the mean and standard deviation of the groups, and the Kolmogorov-Smirnov (K-S) statistical test was used to evaluate the normality of data distribution. Statistical analysis was performed using a t-test to compare the means of the groups. All statistical analyses were performed using SPSS software version 21 at a $P \leq 0.05$.

Ethical considerations. The maintenance and care of experimental animals comply with the policy of the Convention for the Protection of Vertebrates of Iran, which are used for experimental and other scientific purposes, and the protocol was approved by the Ethics Committee of Mazandaran University (UMZ) (No.: 2316813 and date: February 18, 2016).

RESULTS

The changes showed a significant difference ($p<0.001$) between the groups, with the HIF-1α levels of the HIIT group being 23.50% higher than the control one. Independent t-test results showed a significant difference between the HIIT and control groups ($p<0.001$). Apoptosis of lung tissue cells in the training group showed a 57.14% increase compared to the control group. These changes in the two groups' lung tissue weight were insignificant ($p>0.05$) - despite the 18.49% decrease in lung tissue weight in the HIIT group. Also, lung tissue volume in the HIIT group showed a 16.88% decrease compared to the control group, which was insignificant ($p>0.05$).

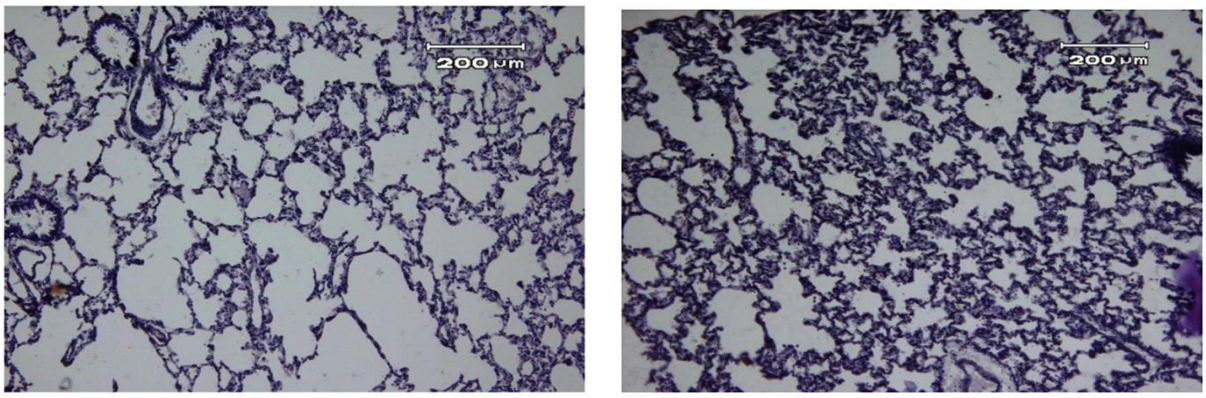
The density of lung tissue (weight to volume ratio) was 4.54% higher in the control group than in the control group; however, this difference was not significant (p>0.05) (Table 1).

Figure 1 showes microscopic representation of lung tissue structure in ats of control group (without HIIT) in which respiratory paremental structure of pulmonary is relatively normal (Figure 1-a), and in rats of HIIT group (in the sixth week) in which a mild interaborbal inflammation and pneumonia and emphysema are visible (Figure 1-b).

Table 1. Variation of Different Indices in Control and Training Groups (Standard Deviation ± Mean)

	Lung tissue weight (gr)	Lung tissue volume (cm^3)	Lung tissue weight/lung tissue volume (gr/cm^3)	HIF-1α (pg/ml)	% Apoptosis Index	Performance (gr*m)
Control	1.46±0.07	2.25±0.5	0.66±0.10	159.4±1.5	2±0.31	177372
Training	1.19±0.17	1.87±0.25	0.63±0.06	**196.9±4.6€**	5±0.44€	**653006€**

€: Significant differences with control group at p<0.05.



a) **b)**

Figure 1. Microscopic representation of lung tissue structure (H&E staining, magnificent 200). **a.** Rats of control group (without HIIT) with a relatively normal respiratory paremental structure of pulmonary. **b.** Rats of HIIT group with a mild interaborbal inflammation and pneumonia and emphysema.

DISCUSSION

In the present study, high-intensity interval training activity was associated with increased levels of HIF-1α in the lung tissue of rats, which may indicate hypoxia induced by high-intensity interval training in this tissue. HIF-1α has been reported to be present in minimal amounts under normoxic conditions (sea-level oxygen), but increased interval training with the induction of hypoxia in various tissues of the body, including lung tissue, results in decreased cellular oxygenation. HIF-1α must bind to HIF-1β to be able to regulate its function under hypoxic conditions. These interactions can be controlled by oxygen levels (10). Under normoxia, HIF-1α is hydroxylated (11) with prolyl hydroxylase domain (PHD), regulated by the VHL[1] tumor suppressor protein, followed by proteasome degradation. Hypoxia prevents HIF-1α hydroxylation and accumulates this subunit, after which HIF-1α is translocated into the nucleus, dimerized to HIF-1β, and transduced with the PAS domain at the beginning of the target transcription gene (10, 12, 13).

Intense training modulates many factors that may alter apoptosis in various tissues (13). The evidence currently supports exercise-induced apoptosis in skeletal muscles and lymphocytes (14). For example, glucocorticoids, growth factor rejection, reactive oxygen species (ROS), increases in intracellular calcium levels, and tumor necrosis factor (TNF) are some of the signals that induce apoptosis (11).

Some of these agents originate from the extracellular environment (TNF and glucocorticoids)

[1]. Von Hippele Lindau

and interact with intracellular or extracellular proteins that may cause cell death (11). Studies show that increases in glucocorticoid secretion, intracellular calcium concentration, and production of reactive oxygen species occur during intense exercise activities and are responsible for apoptosis (11, 15). Although the exact mechanism by which apoptosis occurs is unclear, it may vary depending on the cell type and the type of stimulation (16). In tissues exposed to specific stresses (calcium, glucocorticoids, and free radicals), apoptosis cell death induced by exercise may naturally remove damaged cells (17).

The most crucial reason tissues develop hypoxia is usually inflammation, inadequate circulation, or a combination of these factors (18). Hypoxia develops in areas affected by inflammation (18). In such a context, conditions for the growth of tumors are increased, and glucose concentrations are reduced. Common and dangerous infections can lead to poisoning and destruction of structures and organs that are either acutely or chronically exposed to hypoxia (19).

When cells are exposed to oxygen deficiency, the protective adaptation mechanism initiated by HIF-1α alone is insufficient, so apoptosis occurs (18). Lack of oxygen can cause apoptosis caused by high calcium concentrations in the mitochondrial membrane, leading to the release of cytochrome C (20). Most of the direct induction of hypoxia leading to apoptosis inhibits the electron transport chain in the inner mitochondrial membrane; lack of oxygen inhibits the transport of protons, thereby reducing the membrane potential (20). Depleting mitochondrial-derived ATP activates Bax or Bak, leading to the release of cytochrome C into the cytosol (20). Thus, fibroblasts of mice lacking the Bax and Bak genes are resistant to apoptosis resulting from oxygen deprivation (21).

In addition to energy deprivation, radical formation, primarily reactive oxygen production (ROS), causes hypoxia-induced apoptosis. ROS-activated cascades in human neuroblastoma cells have been reported to differ from classical mitochondrial apoptosis (21). In this case, without cytochrome c in response to oxygen deficiency, the initiator caspase-9 directly activates caspases-3 and -12 (22). After caspase-9 depletion, mitochondrial permeability increases and activates Apaf-1 (22). The effects of hypoxia proapoptosis, cells become resistant to apoptosis during hypoxia(23); Dong et al. showed that cells treated with a potent apoptosis inducer were less susceptible to severe hypoxia (near 0% oxygen), as long as the oxygen level was normal (24).

Hypoxic cell death resistance is present at least in either mitochondrial or cytosolic levels. In cells treated with estrosporin (induced apoptosis resistance), Bax pro-apoptotic protein transport to the mitochondria is suppressed during hypoxia. Bax accumulation in mitochondria causes the release of cytochrome C into the cytosol, which is strongly reduced in a hypoxic medium. This cascade leads to cell death. Bax translocation is suppressed due to an increase in the concentration of inhibitor of apoptosis protein 2 (IAP-2) (24). Resistance to apoptosis disappears by reducing access to IAP-2 from immune lesions. Along with factors that inhibit BAK translocation and maintain mitochondrial integrity, IAP-2 may facilitate cell survival during hypoxia (23).

HIF-1α can increase the potency of the p53 tumor suppressor gene product. As a result of biological stress or DNA damage, p53 can regulate programmed cell death by regulating proteins such as Bax or inhibit the growth mediated by p21 (23). HIF-1α has recently been shown to bind directly to the ubiquitin ligase mdm2 p53 both in vivo and in vitro (25). However, another report showed that p53 binds directly to the ODD domain of HIF-1α. HIF-1α interacts with wild-type p53, not with the p53 mutation tumor (25). It may indicate a difference in the behavior of HIF-1α under physiological versus pathological conditions.

CONCLUSION

According to the present study results, high-intensity interval training enhanced athletic performance, the level of HIF-1α in lung tissue increased, and the rate of apoptosis rose too. Increased interval training appears to increase lung tissue apoptosis despite improving training performance. However, it is anticipated that apoptosis of lung tissue, following hypoxia-inducible factor (HIF)-1, is due to tissue oxygen depletion resulting in high-intensity interval training.

APPLICABLE REMARKS

- High-intensity interval training (HIIT) can induce lung tissue but increases the performance in male Wistar rats.

AUTHORS' CONTRIBUTION

Study concept and design: Saber Niazi, Shadmehr Mirdar. *Acquisition of data:* Gholamreza Hamidian. *Analysis and interpretation of data:* Saber Niazi, Seyed Morteza Tayebi. *Drafting of the manuscript:* Saber Niazi, Seyed Morteza Tayebi. *Critical revision of the manuscript for important intellectual content:* Pablo B. Costa. *Statistical analysis:* Saber Niazi. *Administrative, technical, and material support:* Saber Niazi, Gholamreza Hamidian. *Study supervision:* Saber Niazi, Shadmehr Mirdar.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this study.

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