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ORIGINAL ARTICLE

Administration of vitamin D and aerobic training: recovery of lung apoptosis markers in male rats exposed to hydrogen peroxide

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Abstract

Background Apoptosis is one of the indications of programmed cell death (PCD) and is known as a physiological event in multicellular organisms. This study was designed to determine the effect of aerobic training alongside vitamin D supplementation on lung cell apoptosis in male rats exposed to hydrogen peroxide (H_2O_2).

Methods 48 male rats were assigned into six groups: H_2O_2, (H); $H_2O_2 + D_3$, (HD); H_2O_2 + training, (HE); $H_2O_2 + D_3 + E$, (HDE); dimethyl sulfoxide, (DMSO) and control intact. 1 mmol/kg of H_2O_2 was injected three times per week. The exercising rats performed the program on a rodent's treadmill for 8 weeks, 5 days a week. The HD and HDE groups received a daily dose of 0.5 µg for 8 weeks. The lung tissue was exposed and stored at − 80. Then, the RT-PCR method was employed to examine the gene expressions of BAX, BCL2, Caspase-3 and Bcl-2/Bax ratios.

Results Results indicated that training, as well as a combination of training and vitamin D had a significant effect on BAX, BCL_2 and Bel_2/Bax ratio in case of H_2O_2 toxicity. The training and vitamin D groups both had no significant effect on Caspase-3 gene expression.

Conclusion Based on the results of this research, it can be concluded that regular aerobic training alongside consumption of D_3 might result in significant alteration of the genes involved in apoptosis caused by H_2O_2 presence in lung tissues.

Keywords Apoptosis · Oxidative stress · BCL_2 · BAX · Caspase-3 · Aerobic training · Vitamin D

Introduction

The toxic nature of environmental pollutants and their accumulation in food chain is one of the main biological challenges and health concerns of modern societies. These pollutants find their way into the bloodstreams through the respiratory system and result in the rise of various free radicals that seriously harm human tissues [1]. In addition to these health risk factors, the lungs are constantly exposed to the presence of oxidants such as cigarette smoke, mineral dust, ozone and harmful rays. The apoptosis inflammatory cells can improve or aggravate the damage inflicted on lungs depending on cell types [2, 3]. Majority of the active oxygen and nitrogen types are likely to be produced by phagocytes or polymorphic cells and alveolar, branches or endothelial cells within the lung. There is clear evidence indicating oxidative stress plays a significant role in pathogenesis disease within the lungs [4, 5].

Increase in the level of oxidant and a decrease of defensive antioxidant can lead to the progress of unknown pulmonary fibrosis [6, 7]. Since gaseous exchanges occur within the lung, this organ may become specifically adaptive to prevent damages caused by different types of oxygen [7–9]. The damage caused by oxidative stress is present in numerous diseases including neurobiological diseases, diabetes, atherosclerosis, arthritis, inflammation and most importantly many types of cancer [10, 11]. The condition of oxidative stress with an increase in oxygen types and DNA oxidative damage can act as a trigger to start the apoptosis condition [12]. There are reports showing a wide spectrum of agents which cause apoptotic cell death even though their effectiveness to produce such outcomes depends on condition and types of cells [13]. There are known proteins involved in the molecular path which result in apoptosis, some of which advance apoptosis whereas some prevent the condition [14, 15].

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The protein which is a member of Bel-2 (B-cell lymphoma 2) is likely the most important protein involved in regulating the apoptosis process. Probably the ratio of Bcl-2/Bax (Bcl-2-associated with X Protein) has the closest association with the apoptotic death or life of cells [16, 17]. Activation of the family members of BCL-2 such as Bax and Bak causes the increase of mitochondria outer membrane [18]. Vitamin D plays a significant role in maintaining the homeostasis of different tissues including skeletal muscle, smooth muscle of vascular walls, myocardium and endothelium [19]. Lack of consumption of vitamin D-enriched food lowered the capacity of individuals deprived of sufficient sunlight to synthesize vitamin D, while a higher utilization of vitamin D by the organism due to consumption of certain medications such as corticosteroid can influence the level of vitamin D in respiratory patients as well as improve their lung functions [20].

It is known that all types of aerobic as well as anaerobic trainings have the potential to increase oxidative stress in human and animal models as well depending on the mode, intensity, duration, and diet [21]. A low level of ROS in skeletal muscles has a significant effect on producing forces within the muscles, while a high level of ROS production can cause oxidative damages and hence reduce muscle force production [22]. Results of studies have demonstrated that intense aerobic training may be associated to some damages. In general, acute intensive eccentric training is linked to an increase in pro-apoptosis phenotype and DNA destruction, whereas regular physical activity is associated with an anti-apoptotic condition [23]. In fact, the preferred type of physical activity is regular aerobic training (AET), since it results in improvement and maintenance of cardiovascular and respiratory fitness required to preserve a healthy lifestyle [24].

It is beneficial to understand how it influences oxidative stress pathways which disturb lung functions and to find treatment strategies to handle them. However, mechanisms which activate vitamin D are still unknown and there are no research findings on studying the effect of vitamin consumption alongside aerobic training on apoptosis markers of lungs in case of toxicities. Thus, this research was designed to determine the response of oxidative stress to training in a well-controlled lab investigation; and to determine the role of vitamin D supplement on apoptosis markers. For this purpose, the influence of 8 weeks of aerobic training and vitamin D supplementation on lung cell apoptosis and anti-apoptosis indices of male Wistar rats exposed to hydrogen peroxide was examined.

Material and methodology

Lab animals

In experimental trials, 48 male Wistar rats of 220 ± 20 g weight and 8–10 weeks of age were purchased from the animal center of Shiraz University and were transferred to Kerman University of Medical Sciences. These animals were randomly placed in special 16 × 30 × 42 cubic-sized polypropylene cages. They were kept in standard conditions and controlled temperature (22 ± 2 °C) and at 12 h of daylight and 12 h nighttime (beginning at 7 AM until 7 PM). The animals had free access to food and water (prepared by Pars Co. food for animals, Tehran, Iran). One week after their transfer to experimental environment for purposes of familiarization, forty-eight rats were randomly assigned into six groups, taking part in training, vitamin D diet and stress-inducing conditions, that are as follows: Control, H_2O_2(H), H_2O_2+ (HD)D_3, H_2O_2 + training (HE), $H_2O_2 + D_3$ + E(HDE) and dimethyl sulfoxide (DMSO). All procedures involving animal experimentations were carried out in strict accordance to US Institute of Animal Research guidelines and were approved by the Animal Care and Usage Committee of the University of Kerman and Ministry of Health and Medical Education adhering to ethic no. IR.KMU.REC.1396.1562.

H_2O_2 toxicity induction

The experimental groups assigned to induce toxicity condition received 1 mmol/kg dosage of H_2O_2 and were injected peritoneum [25, 26] three times per week in alternate days.

Aerobic training protocol

The exercising rats performed the designed aerobic training program on rodent's treadmill for 5 days a week and for a total duration of 8 weeks. The rats in these groups were trained for 2 weeks on treadmill at the speed of 10–15 m/min for 30 min to learn how to complete the training protocol. The slope of treadmill was steady at 10°, but the speed and duration of the training gradually increased from 8 m/min for 30 min in 1st week, to 12 m/min for same duration in 2nd week, to 16 m/min for 45 min in 3rd week, and to 20 m/min for 45 min in 5th week. During fifth to eighth weeks, the speed remained at 20 m/min for 60 min. To warm up and cool down, running was performed for 5 min at 40% of their VO_{2max} [27].

Vitamin D administration

The injection of vitamin D_3 was performed on HD and HDE groups. These rats received a dose of 0.5 µg vitamin D peritoneum daily for 8 weeks [28]. The D_3 vitamin dose with 300,000 UI/ml concentration was purchased from the Caspian Co. Tehran, Iran under the generic name of DITHRECOL. Normal saline was added for purpose of preparing the appropriate dose and dimethyl sulfoxide (DMSO) was used to make vitamin D soluble in normal saline. To

determine if this solvent had any effects, a group named DMSO only received a daily dose of this solvent.

Collecting lung samples

24 h after the last training session and following 12 h of fasting, the animals' lungs were exposed to avoid extra production of internal ROS [29]. The lung tissue was removed, immediately rinsed with 0.1% phosphate buffer and kept in RNA Later solution (Ambion, L/N: 1,206,029, USA) prior to RNA extraction for measuring Bax, Bcl2, and Caspase 3. This solvent is used to fix and protect the tissues' cell RNA.

Processing the tissue and RNA extraction

The entire RNA was extracted from 30 mg of tissue (wet weight) using mini kit QIAGEN (kit, QIAGEN, Germany). Tissue was also homogenized using rotor state (Tissue Rupture, 230V, 50–60 Hz_ QIAGEN, Germany), then by adding 590 ml of RNase free water and 10 ml of proteinase K to incubate, the homogenized solvent was kept at − 55° for 20 min. Afterwards, it was centrifuged for 25–30 min and transferred to a new tube. Following this process, pure ethanol was added to half of the volume (usually 450 ml) containing RNA. After washing with buffer RW1, it was directly transferred to a storage containing special filter; finally 30 ml of free water was added and total RNA was collected and kept in − 20° centigrade.

cDNA synthesis and mRNA expression analysis by real-time PCR (qPCR)

It is necessary to transform RNA samples into cDNA to perform two-stage PCR. Reverse transcription to cDNA is performed using qRT-PCR high-capacity RNA to cDNA kit. This kit contains reactors for reverse buffer transcription and enzymes that convert the total RNA to single-string cDNA. Considering the instruction provided by manufacturer, total RNA is converted to cDNA by adding 1000 mg RNA (10 ul of buffer RT and 1 ml of RT). Specific amplification fragments of DNA/RNA two-step real-time qPCR (quantitative polymerase chain reaction technique) were used to calculate gene expression during the PCR amplification process applying TaqMan® reagent. In order for reaction to take place, 50 ng cDNA in 1 µl, 5 ml of main buffer, 1 µl of herprot gene and 3.5 µl of water without RNase were loaded in special holes. The total volume of mixed PCR per hole was 10 µl. All experiments were conducted in three biological replicates in a Step One-Plus real-time PCR machine (Applied Biosystems, USA). The real-time PCR program includes reverse transcription at 48 °C for 15 min, activation of ampli-Taq gold DNA polymerase at 95 °C for 10 min, denaturation at 95 °C for 15 s, and annealing at 60 °C for 1 min. Denaturation and annealing steps were performed for 40 cycles. Beta-actin and Gapdh were used as housekeeping genes. The fold changes of each target per average Beta-actin and Gapdh were calculated and considered as mRNA expression levels of the target gene. Data were analyzed according to comparative CT ($2^{-\Delta\Delta Ct}$) method [30] where amplification of the target and the reference genes was measured in the sample and the reference. The sequences of primers are presented in Table 1.

Statistical analysis

All data were presented as mean ± standard error of mean (SEM). Shapiro–Wilk test was applied to evaluate data normality and homogeneity distribution. One-way ANOVA with Tukey's post hoc test were used to determine pairwise difference and the level of significance was set at $p < 0.05$. Graph Pad prism software was employed to analyze data. Levene's test confirmed the homogeneity of variances. The test of hypothesis was performed applying parametric tests including one-way analysis of variance (ANOVA) when the variables showed normal distribution, then Tukey's post hoc test was used to locate the differences. Two-way ANOVA was also utilized to simultaneously test more than one independent variable.

Table 1 The sequence of primary nucleotide

Gene	Forward primer	Reverse primer	Amplicon length (bp)
Bax	ccccgtgagg gccgcacgtc tgcggggagt cacgtgaccg	mdgsgdhlgg ggptsseqim ktgafllqgf iqdraermag	63
Bcl-2	cctcatgaaa taaaaagctg aaaggaattt gaataaaaat	maqagrtgyd nreivmkyih yklsqrgyew dtgdedsapl	104
Casp-3	gggatcaaag cttagtgtcc tgaggtgcgg agcttggaac	mdnnetsvds ksinnfetkt ihgsksmdsg iyldssykmd	93
Beta–actin reference gene Actb	gtcgagtccg cgtccacccg cgagtacaac cttcttgcag ctcctccgtc gccggtccac	mdddiaalvv dngsgmckag fagddaprav fpsivgrprh qgvmvgmgqk dsyvgdeaqs	91
Gapdh reference gene	ggggctctct gctcctccct gttctagaga cagccgcatc ttcttgtgca gtgccagcct	mvkvgvngfg rigrlvtraa fscdkvdiva indpfidlny mvymfqydst hgkfngtvka	174

Results

The result of one-way ANOVA indicated that there was a significant difference between the mean of BAX gene expression ($P=0.001$). Tukey's post hoc test results showed that the expression of BAX gene was significantly higher than the solvent ($P=0.01$) and the control ($P=0.002$) group. The result of two-way ANOVA for training ($F=25.24$, $P=0.01$, $\mu=0.759$) and vitamin D ($F=24.84$, $P=0.01$, $\mu=0.756$) indicated that both training and vitamin D ($F=10.29$, $P=0.012$, $\mu=0.563$) had a significant main effect as well as interactive effect on BAX gene expression. There was a significant decrease in gene expression in these conditions (Fig. 1).

The expression of BCL-2 in the group which received hydrogen peroxide significantly decreased compared to the solvent ($P=0.046$ (and the control group ($P=0.043$). In the condition with 1 mmol/kg hydrogen peroxide, training resulted in a significantly higher expression of BCL-2 gene ($F=5.17$, $P=0.05$, $\mu=0.393$). The vitamin D group also resulted in a significantly higher expression of BCL-2 gene ($F=6.05$, $P=0.039$, $\mu=0.431$), but there was no significant interaction for training and vitamin D supplement ($F=4.11$, $P=0.077$, $\mu=0.340$) (Fig. 2).

Results indicated that Bax/Bcl-2 ratio was significantly less than the solvent ($P=0.01$) and the control group ($P=0.01$). In addition, this difference was significantly lower in the solvent group compared to the control group ($P=0.006$). Also, the training group, when received hydrogen peroxide, showed a significantly less ratio of Bax/Bcl-2 ($F=12.93$, $P=0.007$, $\mu=0.618$). Vitamin D group alone, had also a significant effect on the gene expression ratio of Bax/Bcl-2 ($F=15.29$, $P=0.04$, $\mu=0.657$). Moreover, there was an interactive effect present for simultaneous application of training and vitamin D supplementation on this variable ($F=3.91$, $P=0.013$, $\mu=0.329$) (Fig. 3).

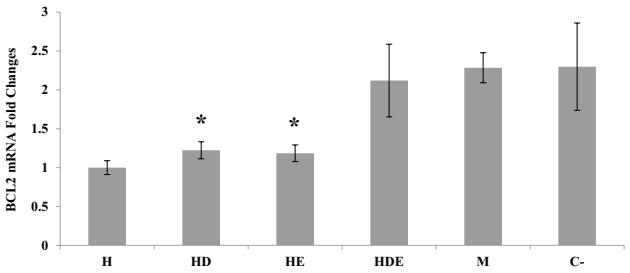

Fig. 2 BCL-2 gene expression in experimental groups. *H* hydrogen peroxide, *HD* hydrogen peroxide + Vitamin D, *HE* hydrogen peroxide + aerobic training, *HDE* hydrogen peroxide + Vitamin D + aerobic training, *M* dimethyl sulfoxide, *C* control intact. Data were expressed as mean ± SEM

Hydrogen peroxide caused a significant increase in the level of Caspase-3 ($P=0.01$). The result of one-way ANOVA showed that expression of Caspase-3 gene in H_2O_2 group was significantly higher than solvent ($P=0.01$) and control ($P=0.01$) groups. On the other hand, when 1 mmol. kg of hydrogen peroxide was used, training had no significant effect on Caspase-3 ($F=0.82$, $P=0.391$, $\mu=0.093$). Additionally, Vitamin D supplement ($F=2.36$, $P=0.162$, $\mu=0.228$) and its interaction with training also had no significant effect on the level of Caspase-3 ($F=0.56$, $P=0.475$, $\mu=0.066$) (Fig. 4).

Discussion

This research is one of the first investigations to examine the effect of training, vitamin D and induced oxidative stress on lung BAX, BCL, CASPASE gene expressions. Previous researchers mainly examined the heart and skeletal muscles. Regular training program increases the resistance of respiratory muscles and results in the improvement of respiration function and increases maximum exhale output [31]. Based

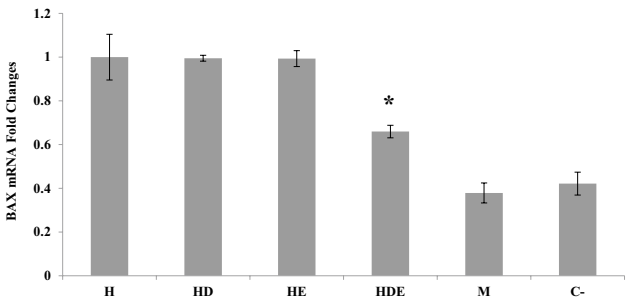

Fig. 1 Bax gene expression in experimental groups. *H* hydrogen peroxide, *HD* hydrogen peroxide + Vitamin D, *HE* hydrogen peroxide + aerobic training, *HDE* hydrogen peroxide + Vitamin D + aerobic training, *M* dimethyl sulfoxide, *C* control intact. Data were expressed as mean ± SEM

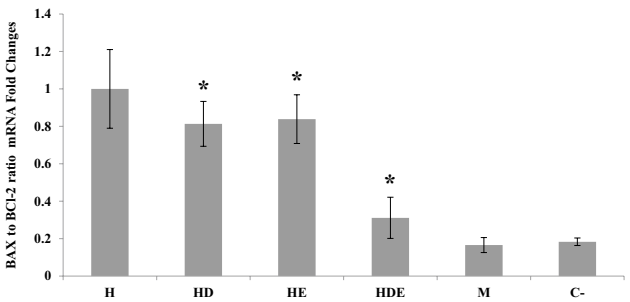

Fig. 3 Ratio of BAX/Bcl-2 gene expression in experimental groups. *H* hydrogen peroxide, *HD* hydrogen peroxide + Vitamin D, *HE* hydrogen peroxide + aerobic training, *HDE* hydrogen peroxide + Vitamin D + aerobic training, *M* Dimethyl sulfoxide, *C* control intact. Data were expressed as mean ± SEM

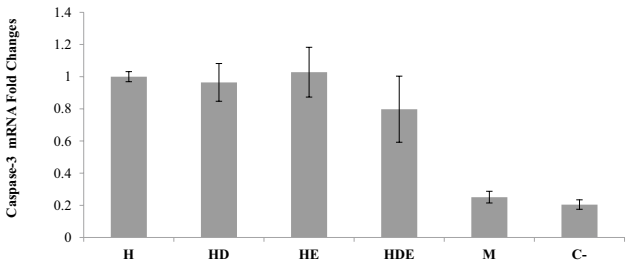

Fig. 4 Caspase-3 gene expression in experimental groups. *H* hydrogen peroxide, *HD* hydrogen peroxide + Vitamin D, *HE* hydrogen peroxide + aerobic training, *HDE* hydrogen peroxide + Vitamin D + aerobic training, *M* dimethyl sulfoxide, *C* control intact. Data were expressed as mean ± SEM

on the results of this study, participation in regular aerobic training or taking vitamin D supplement alone significantly improved the lung apoptosis and anti-apoptosis indices; however, there was a different result for the interaction of these two variables in the expression of apoptotic genes. The interaction of vitamin D and aerobic training had a significant decreasing effect on level of BAX expression compared to the control group; all groups showed lower level of expressions; however, the interactive application of vitamin D and aerobic training neither had any significant effect on Caspase-3 and BCL anti-apoptotic gene expression nor on BCL-2/BAX ratio.

Findings of present research indicated that oxidative stress induced by the application of hydrogen peroxide resulted in an increased level of BAX. This result was expected since it has been shown before that oxidative stress is one of the triggering agents to activate apoptosis [32]. Training program and vitamin D decreased the oxidative pressure of BAX. BAX protein may be the cause of damage to the outer mitochondrial membrane; they eventually result in the distribution of cytochrome c into cytosol. In addition, it has been demonstrated that NAX is linked to BCL-2 and prevents it from doing its anti-apoptotic function [33].

In contrast, results of another research have indicated that participants in regular aerobic training could have the same lung function [34] or have no improvement in their lung function at all but only in their cardiorespiratory capacity [35]. In that research, it was demonstrated that increased BCL-2 and decreased BAD in general controlled the apoptosis; however, findings of present research did not support findings of Phaneuf that claimed immediately after exercise session BCL-2 decreased while level of BAX increased [36]. The reason behind these contradictory findings may be attributed to the length of training programs. The intensity of exercise may have a role in triggering the immune system the same, as it is triggered in diseases such as cancer and AIDS, which may cause disruption of its function or may suppress the response of the immune system [37].

Lung inflammation causes infiltration of inflammatory cells into pleural, alveolar and intercellular spaces. Increase in aerial spaces and inter tissue spaces is visible during the acute lung inflammation. Afterwards, inflammatory cell starts to release poisonous factors such as protease and free radicals into the inflamed region which harms the parenchyma cells causing apoptosis [38] or develops asthma during adolescence when exposure to air pollution [39]. The result of one study on changes in level of BCL2 in response to training and vitamin D supplement alone indicated that either of these two variables could cause a significant increase in BCL2 gene expression, yet, no significant interactive effect was found.

Considering the fact that BCL2 prevents the increase in BAX, it seems that the increase in BCL2 is one of the mechanisms that suppresses Bax changes [40]. Results of studies showed that increase in BCL-2 as one antioxidant agent consolidates the mitochondrial wall, suppresses BAX, prevents the release of cytochrome c, regulates released calcium from sarcoplasm, decreases the ROS produced during physical activities and increases cell immunity which prevents cell apoptosis due to stress [41]. It has been shown that BCL-2 itself does not act as an antioxidant, but it may have an indirect effect on the increase of antioxidant activities within the cell. Therefore, the increase of BCL-2 protein allows the cells to deal with free radicals more effectively; such changes occur as a result of increase in antioxidant enzymes of defense in immune system [12, 42]. It is well documented that active free radicals via smoking also are capable of oxidizing the biomolecules and may reorient the gene expression to cause cancer [43]. In the present research, it is shown that induced oxidative stress resulting from the injection of hydrogen peroxide would increase the pre-apoptosis factors in tissues, and as a result would expose the lungs to apoptosis. Phaneuf et al. reported that sport training results in apoptosis that is a natural process for the damaged cell and no noticeable inflammatory reaction occurs in the cell. This process is an assurance for the natural function of the body [36, 42]. Moreover, an intensive exercise program results in disturbance of homeostasis and is associated with an increase of oxidative stress [44].

Fisher et al. demonstrated that intensive interval training intensifies oxidative stress in cells [45]. The free radicals of oxygen that resulted from oxidative stress can damage DNA in many different organs including lungs and can lead to apoptosis. In addition, air pollution causes lung aggressor as it was associated with lung cancer and cardiopulmonary mortality [46]. During the intensive physical activities, there is an increase in free radicals that cause oxidative stress with indices such as lipid and protein peroxidation, as well as a decrease in superoxide dismutase and catalase [47]. It is logical to take measures to prevent such production or to minimize it. While such a condition is accurate in terms of

intensive training programs, yet mild or moderate physical activities seem to decrease apoptosis in different tissues [48]. The contradictory findings in this regard may be attributed to different patterns of training programs. Based on the result of this research, aerobic training program had a significant effect on Bcl/BAX. Vitamin D supplement also had a significant effect on Bcl-2/Bax gene expression. The result of some studies showed that the balance between pre-apoptosis and anti-apoptosis proteins including Bcl-2/Bax ratio determines the senility rate of cells to the signals for programmed cell death apoptosis [49]. Since an increase in Bax protein helps the start of apoptosis and an increase in Bcl-2 prevents its process [50], the increase in this ratio forces the cell towards apoptosis [51]. On the other hand, 3% of human genome is under the control of 1 and 25-hydroxyvitamin D and nearly all human cells has receptors for vitamin D; therefore, the function of this micronutrient is greatly more than merely calcium regulation and phosphorus metabolism [52]. Thus, decrease in ratio of Bcl-2/Bax observed in the present research may be attributed to the fact that aerobic training and vitamin D functioned as antioxidant and probably regulated the factors that lead to Bax and p53 and prevented the path for the cytochrome C [53] and suppressed external agents including ROS and TNF-α [54]. This occurrence increased the ratio in favor of survival—a process that may be considered as anti-apoptotic for the lung tissue despite the decrease in Bcl-2/Bax ratio that has been reported after an intense aerobic exercise [55]. There was no significant interactive effect for the aerobic training and vitamin D supplement on Bcl-2/Bax gene expression in the present research, probably due to low levels of induced apoptosis caused by H_2O_2 or due to the fact that participants in this research had 2 months of regular training program prior to this experiment and such a condition is associated with mechanisms that eliminate free radicals and improve the state of cell destruction [56].

Aerobic training and vitamin D supplement did not cause significant changes in Caspase-3. This may be an indication of the protective role of aerobic training in process of tissue apoptosis. Normal cells have specific protective factors which they use against caspases. The decrease in apoptosis process induced by aerobic training shows that regular aerobic training during oxidative stress can function as a protective strategy to prevent cell apoptosis in lungs. Several mechanisms have been suggested for such protective role including altered anti-protein expression and pro-apoptosis, increased mitochondrial biogenesis and improved mitochondrial function, and increased level of antioxidant or fewer types of reactive oxygen [23]. In vitamin D group, the ratio of caspase-3 expression was less than control group. On the other hand, results of present research showed that aerobic training combined with usage of vitamin D while receiving hydrogen peroxide had no significant effect on caspase-3 gene. Yet, there is abundant evidence indicating that an ordinary diet may have considerable antioxidant effect on decreasing free radicals. There is evidence about training function or adaptability benefits [57–59] and there are a limited number of research reports to show the antioxidant benefits of supplementary diet [60] and several research results have shown no beneficiary effect for such interventions [61, 62]. It has been suggested that supplementary antioxidant substances are effective in improving performance provided that the internal sources are depleting and once the store is saturated, there will be no further benefits [63]. In addition, it has not been seen that after an intensive training program, antioxidant supplementation is beneficiary during the recovery period [64].

Conclusion

In summary, results of this study showed that great oxidative stress caused by injection of hydrogen peroxide can result in lung inflammation which can result in alteration of gene expression of BCL2, BAX, Caspase-3, Bcl-2/Bax which all play a significant role in lung tissue. In addition, this type of regular aerobic training performed on treadmill and injection of vitamin D as antioxidants could control oxidative stress. The interaction of vitamin D and aerobic training plays a protective role when apoptosis is induced in lung tissues. As oxidative stress due to reaching pathologic stages such as smoking and air pollution could be accrued at younger ages, regular aerobic training with sufficient intensity and duration could be recommended as a non-drug intervention to decrease lung apoptosis. However, further carefully designed research taking into consideration other factors involved in the apoptosis process needs to be conducted. The researchers also suggest more regular aerobic training involving additional indices of apoptosis to be performed to examine internal and external paths.

Limitation of study

This study is an animal model, hence certain limitations are unavoidable. It should be noticed that we wished to investigate responses of lung to different ventilator strategies independent of changes in cardiac output that resulted from different ventilator patterns; yet, we were unable to measure lung function as a second outcome and a marker to directly illustrate oxidative stress.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent For this type of study formal consent is not required.

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