

Effect of Aerobic Exercise and Vitamin D Supplementation on Expression of Apoptosis-Regulatory Genes in Lung of Rats Poisoned with Hydrogen Peroxide

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Received: 13 Dec 2018

Revised: 02 Jan 2019

Accepted: 20 Jan 2019



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ABSTRACT

Background and Objectives: Apoptosis is essential for the survival and normal functioning of multicellular organisms, yet any interruption in this process could be detrimental. Increased production of reactive oxygen species and oxidative stress are key factors affecting apoptosis. Our objective was to determine the impact of exercise with and without vitamin D supplementation on expression of FLIP, Fas, and caspase 8 in lung of rats poisoned with H₂O₂.

Methods: Forty-eight adult male rats were randomly divided into six groups: (C), (H), (HD), (HE), (HDE) and dimethyl sulfoxide. Groups H, HE, HD and HDE received 1mmol/Kg intraperitoneal injection of H₂O₂. HE and HDE groups ran on treadmill for eight weeks. Expression of FLIP, Fas and caspase 8 was measured in lung tissues using RT-qPCR. Statistical analysis of data was carried out using SPSS 22 at significance level of 0.05.

Results: Vitamin D supplementation caused a significant decrease in expression of Fas (P=0.014) and caspase 8 (P=0.016) compared to the control group. However, it significantly overexpressed FLIP in the lung tissues compared to the control group (P=0.005). Exercise with and without vitamin D supplementation had no significant effect on the expression of the apoptosis regulatory genes.

Conclusion: Our results show that VD exerts protective effects on lung tissue by regulating apoptotic factors. Aerobic exercise alone and combined with VD has no significant effect on the apoptotic factors. These results indicate that VD supplementation can reduce lung injury under oxidative stress conditions.

KEYWORDS: Apoptosis, Vitamin D, Exercise.

INTRODUCTION

Respiratory diseases are one of the most common causes of human death worldwide (1). Approximately 1.2 million people are diagnosed with lung cancer every year (2). It has been proposed that reactive oxygen species (ROS) may be involved in lung injury (3). ROS can induce apoptosis by activating cellular apoptotic pathways or causing damage to cellular components such as DNA, mitochondria and lysosomes (4).

Vitamin D (VD) can reduce risk of cancer by regulating cell proliferation and differentiation and inhibiting angiogenesis (5). Endurance exercise at moderate intensity reduces oxidative stress and muscle damage, while intensive endurance exercise have adverse effects on the body's antioxidant defense system by increasing the production of free radicals (6). VD also exerts pro-differentiating and anti-metastatic effects by regulating transcription of more than 60 genes (5). VD protects epithelial cells against H₂O₂-induced oxidative stress by inhibiting apoptosis and production of superoxide anion (7). VD can induce apoptosis in cancer cells through p53-dependent and independent pathways (1).

In recent years, several studies have investigated the effects of different types of training on apoptosis. Unlike intestine exercise, performing moderate and sustained exercise can decrease apoptosis in different tissues (8-10). Oxygen free radicals can damage cellular structures and DNA, which may lead to apoptosis (11). Regular physical activity strengthens the antioxidant defense system and protects the body against free radicals-induced damage. It is well-established that regular physical activity has an important role in prevention or control of some lung diseases (12). In this study, we determine effects of aerobic exercise with and without VD supplementation on expression of apoptosis regulatory genes in rats poisoned with H₂O₂.

MATERIALS AND METHODS

The study was carried out on 48 adult male Wistar rats aged 8-10 weeks (weighting 220±20 g). The rats were kept at 22 °C on a 12:12 h light/dark cycle. All experiments on the animals were approved by the ethics committee of the Kerman University of Medical Sciences (ethical approval code: IR.KMU.REC.1396.1562). The rats were

randomly and equally divided into six groups: control group (C), poisoned with hydrogen peroxide (H), hydrogen peroxide and VD (HD), hydrogen peroxide and aerobic exercise (HE), hydrogen peroxide, VD and aerobic exercise (HDE) and dimethyl sulfoxide (DMSO) with saline. Animals were weighed every two weeks and their food consumption was monitored on a daily basis. Rats in the HD, HE, H and HDE groups received intraperitoneal injection of 0.1 mg/Kg H₂O₂ (Merck, Germany) three times a week. Rats in the HD and HDE group received daily intraperitoneal injection of 0.5 µg/Kg body weight VD (300,000 IU, Dithrecol, Caspian Tamin Co., Iran) for eight weeks (13). Normal saline was used to dilute VD and DMSO was used to dissolve VD in saline.

Subjects in the HE and HDE group ran on a rat running wheel for eight weeks. Table 1 shows the duration and speed of exercise over the eight-week intervention (14).

Rats began running at speed of 15 m/min for 2 minutes. The speed of running wheels was increased by 1.8 m/min every 2 minutes until the rats were unable to run. VO₂max values were calculated based on the correlation between speed of running wheel and rats' VO₂max (15). The VO₂max values were determined at baseline, at the end of the fourth week and at the end of eighth week (16).

Twenty four hours after the last training session and following 12 hours of fasting, the rats were sacrificed and lungs were exposed to avoid extra production of internal ROS (17). The lung tissue was removed, immediately washed with 0.1% phosphate buffer and fixed in RNAlater solution (Ambion, L/N: 1206029, USA). The tissue samples were homogenized using a rotor stator homogenizer (Tissue Rupture, 230V, 50-60 Hz, QIAGEN, Germany) and then kept at -55 °C for 20 minutes. Total RNA was extracted from 30 mg of wet tissue using RNeasy Mini Kit (QIAGEN, Germany). After centrifugation for 25 to 30 minutes, the mixture was transferred to a new tube and treated with pure ethanol. After washing with buffer RW1, the mixture was directly transferred to a storage containing a special filter. After adding 30 µl of water, total RNA was collected and kept in -20 °C. The extracted RNA solution was treated with DNaseI enzyme. Quality of the extracted RNA was measured by reading absorbance at

260/280 nm and electrophoresis on 1% agarose gel. For synthesis of cDNA, 5 µg of each RNA sample were mixed with oligo-dT primers (Parstous Biotechnology) and reverse transcriptase according to kit manufacturer's instructions (Parstous Biotechnology). The primers were designed using the Primer 3 software. For performing two-stage PCR, reverse transcription to cDNA was performed using Real-Time Quantitative Reverse Transcription PCR (RT-PCR) high capacity RNA to cDNA kit. Total RNA was converted to cDNA by adding 1000 ng RNA (10 µl of buffer RT and 1 µl of RT). All experiments were conducted in triplicate in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). Amplification conditions were as follows: reverse transcription at 48 °C for 15 min, activation of ampli-Taq gold DNA polymerase at 95 °C for

10 min, 40 cycles of denaturation at 95 °C for 15 s and 0 cycles of annealing at 60 °C for 1 min. Beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as the housekeeping genes. Data was analyzed according to the comparative Ct method ($2^{-\Delta\Delta C_t}$) (18).

PCR primers and a TaqMan probe with a dye label (FAM) was ordered from Thermo Fisher Scientific for Fas (Rn00685720_m1), FLIP (Rn01438105_m1), caspase 8 (Rn01440170_m1), beta actin (Rn00667869_m1) and GAPDH (Rn01775763_g1). Data were analyzed with SPSS 22 at significance level of 0.05. One-way analysis of variance was performed for determining the effect of receiving H₂O₂ (1 mmol.Kg). Two-way analysis of variance was used for independent groups to determine effect of exercise and VD supplementation.

Table 1- Duration and speed of exercise over the eight-week intervention period

Week	Speed (m/min)	Time (min)
1	8	30
2	12	30
3	16	45
4	20	45
5	20	60
6	20	60
7	20	60
8	20	60

RESULTS

Fas expression was significantly higher in group H compared to group C ($P=0.0001$). Exercise had no significant effect on expression of the Fas gene ($P=0.598$), while VD supplementation significantly decreased Fas expression ($P=0.205$). Combination of exercise and VD supplementation had no significant effect on Fas expression ($P=0.734$, Figure 1).

Exercise had no significant effect on expression of the FLIP gene ($P=0.863$), but VD supplementation caused a significant increase in FLIP expression ($P=0.005$). Combination of exercise and VD supplementation had no significant effect on FLIP expression ($P=0.531$, Figure 2).

Figure 1- Expression of the Fas gene in different groups

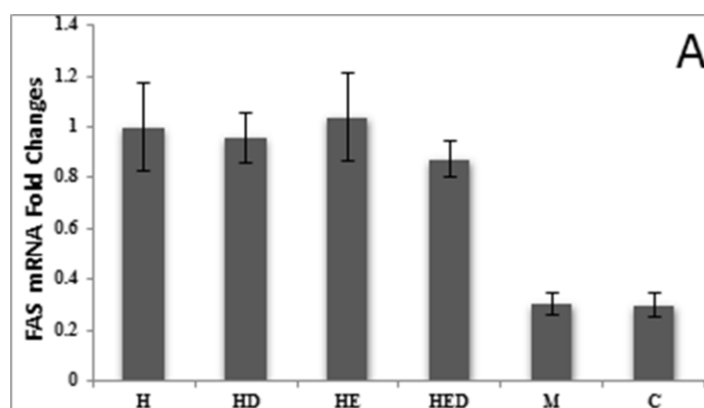
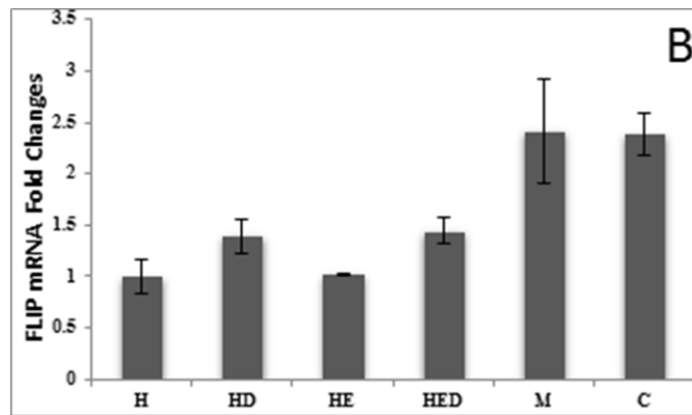


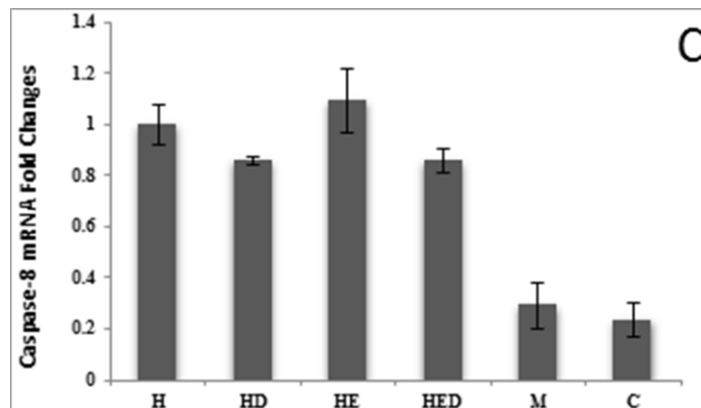
Figure 2- FLIP expression in different groups



Exercise had no significant effect on caspase 8 expression ($P=0.245$), but VD caused a significant decrease in caspase 8 expression

($P=0.016$). Combination of exercise and VD had no significant effect on caspase 8 expression ($P=0.238$, Figure 3).

Figure 3- Caspase 8 expression in different groups



DISCUSSION

Our findings showed that eight weeks of regular aerobic exercise with or without VD supplementation had no significant effect on expression of caspase 8, Fas and FLIP in rats exposed to hydrogen peroxide. In line with our findings, Koopman et al. reported that moderate exercise caused no change in the percentage of apoptotic cells (19). Some studies demonstrated that unlike intensive exercise, moderate intensity exercise causes little or no apoptosis (6,20). According to Dumont et al., Fas (CD95) system is not essential for H_2O_2 -induced apoptosis (21). Several metabolic and hormonal changes may occur during exercise which can result into cell damage or apoptosis in laboratory conditions. For instance, corticosteroids, catecholamines and cortisol can induce apoptosis in peripheral blood lymphocytes in a time-, concentration- and exercise intensity-dependent manner (22). It is possible that the exercise intensity in our study was not high enough to increase level of these hormones to

cause apoptosis. Numerous studies have shown that exercise can alter calcium levels in cytosol, which is an essential intracellular signal that interferes with the apoptotic processes. In addition, increased cytosolic calcium level prior to apoptosis has been detected in some cell types (21). It is likely that the exercise in our study was not intense enough to increase cytosolic calcium level.

It has been demonstrated that aerobic exercise including running can significantly decrease FasL level (6,23,24).

In a study, thirty minutes of daily pedaling on ergometer bike for 12 weeks considerably decreased soluble forms of Fas and FasL (24). There seems to be a direct relationship between concentration of apoptotic mediators and exercise intensity (25,26). The lack of effect on apoptotic mediators could also be due to insufficient amount of H_2O_2 used for the induction of oxidative stress (10). VD has been identified as a strong regulator of growth, differentiation, angiogenesis and

apoptosis (27). In our study, VD supplementation decreased Fas and caspase 8 expression and increased FLIP expression compared to the control group.

Zeng et al. reported that VD supplementation can lower the overexpressed Fas and FasL in hearts of rats with diabetic cardiomyopathy (28). Other studies also reported that calcitriol exerts anti-apoptotic effects through regulation of Bcl-2 (29, 30). Active form of VD can also inhibit FAS- and TNF α -induced apoptosis by lowering LEX-1 transcription, which plays a role in cell survival (31).

Zhang et al. demonstrated that pretreatment with 1,25 (OH) $_2$ D $_3$ decreases apoptosis induction by TRAIL and FasL, while continuous treatment with this compound induces apoptosis in ovarian cancer cells (32). In our study, VD supplementation reduced apoptosis in pulmonary cells through decreasing pro-apoptotic factors Fas and caspase 8 and increasing anti-apoptotic factor FLIP. However, VD supplementation combined with

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aerobic exercise had no notable impact on Fas, caspase8 and FLIP. This could be related to negative effects of oxidative stress and calcitriol on expression of VD receptor (33).

CONCLUSION

Our results show that VD exerts protective effects on lung tissue by regulating apoptotic factors. Aerobic exercise alone and combined with VD has no significant effect on the apoptotic factors. These results indicate that VD supplementation can reduce lung injury under oxidative stress conditions.

ACKNOWLEDGEMENTS

Our deepest gratitude goes to the faculty of physiology at Kerman University of Medical Sciences for their cooperation and support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding publication of this article.

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