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The Effects of Endurance Training and Vitamin D Supplementation on the Bone Resorption Markers in the Rats Exposed to Oxidative Damage Induced by Hydrogen Peroxide (H_2O_2)

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ABSTRACT

Introduction: Various studies have indicated that increased active oxygen species is associated with bone cell damage. The key role of physical exercise and vitamin D supplementation on bone health has been confirmed. The present study aimed to investigate the interactive effects of aerobic exercise with vitamin D supplementation on the bone resorption markers in the rats exposed to oxidative damage induced by hydrogen peroxide (H_2O_2).

Methods: This experimental study was conducted on 60 adult male Wistar rats. The animals were randomly divided into 10 groups of six, including H_2O_2 (1 mmol/kg), H_2O_2 (2 mmol/kg), H_2O_2 (1 mmol/kg) with vitamin D, H_2O_2 (1 mmol/kg) with endurance training, H_2O_2 (1 mmol/kg) with vitamin D and endurance training, H_2O_2 (2 mmol/kg) with vitamin D, H_2O_2 (2 mmol/kg) with endurance training, H_2O_2 (2 mmol/kg) with vitamin D and endurance training, sham (dimethyl-sulfoxide with normal saline), and control. The intervention was performed for eight weeks, and the levels of tartrate-resistant acid phosphatase 5b (TRACP/5B) and N-telopeptides (NTx) were measured using the ELISA assay. Data analysis was performed using the Kolmogorov-Smirnov test, two-way analysis of variance (ANOVA), one-way ANOVA, and Tukey's post-hoc test at the significance level of $P \leq 0.05$.

Results: In the animals administered with 1 and 2 mmol/kg of H_2O_2, no significant effects were observed on the levels of NTx (P=0.76 and P=0.47, respectively) and TRACP/5B (P=0.48). On the other hand, endurance training increased the NTx levels in the rats exposed to 2 mmol/kg of H_2O_2 (P=0.04), while vitamin D had no significant effects on the levels of NTx (P=0.32) and TRACP/5B (P=0.92). In addition, endurance training with vitamin D supplementation had no interactive effects on increased NTx and TRACP/5B in the rats exposed to 1 mmol/kg (P=0.67 and P=0.99, respectively) and 2 mmol/kg of H_2O_2 (P=0.16 and P=0.47, respectively).

Conclusion: According to the results, endurance training could significantly increase the NTx level in the rats exposed to the oxidative damage induced by 2 mg/kg of H_2O_2.

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Introduction

Oxidative stress occurs due to the imbalance between the production of reactive oxygen and nitrogen species and antioxidant defense system of the body. As such, the physiological intracellular redox state depends on the ratio of oxidative stress and antioxidants (1, 2). Reactive oxygen species (ROS) are highly reactive molecules, which consist of a number of diverse chemical species, including radical and non-radical oxygen species, such as superoxide anion (O_2-), hydroxyl radical (OH-), and hydrogen peroxide (H_2O_2) (3).

The controlled increase of ROS levels, particularly H_2O_2, may play a key role in the transmission of intracellular signaling, which regulates numerous fundamental cellular processes, such as proliferation, differentiation, apoptosis, repair processes, and inflammation (3, 4). *In-vitro* evidence suggests that ROS could be involved in the pathogenesis of osteoporosis, which is characterized by increased bone loss

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and the subsequent higher risk of bone fractures (5).

ROS elicit a spectrum of responses, ranging from cell proliferation, growth, differentiation, and arrest to cell death, through activating numerous signaling pathways. Mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinases (ERK1/2), c-Jun-N terminal kinase (JNK) and p38 MAPK are involved in osteoblast or osteocyte apoptosis (1, 6, 7). On the other hand, bone formation markers reflect osteoblast activity. Bone resorption markers are indicative of the enzymes of the osteoblastic cells (e.g., N- and C- telopeptides of type I [N-telopeptides [NTx] and CTx] and 5b isoenzyme of tartrate-resistant acid phosphatase [TRAP5b]) or the proteolytic fragments of bone collagen matrix (e.g., type I collagen) (8). The increased production of the receptor activator of the nuclear factor-kappa B ligand (RANKL) and activation of ERK/NF-κB/TNF/interleukin 6 cause the ROS to inhibit osteoclast apoptosis and promote osteoclastogenesis (9). In addition, RANKL has been reported to suppress the transcriptional activity of FOXOs (10).

FOXOs are the crucial regulators of osteoblast and osteoclast physiology, as well as direct mechanistic links between oxidative stress and skeletal involution (11). Researchers believe that bone remodeling is regulated by various systemic, local, and nutritional factors, such as calcium and vitamin D. However, the contradictory findings in this regard have necessitated further investigations in order to determine the association between the responses of bone markers (e.g., osteocalcin, bone alkaline phosphatase [ALP], and urine N-telopeptide crosslinks) to changes in the vitamin D status (12).

A study in this regard indicated that the administration of 50,000 IU of vitamin D once per week for 8-12 weeks followed by administration once a month could significantly decrease B-cell-specific activator protein (BSAP), while no significant effect was reported on NTx in postmenopausal women with osteoporosis and osteopenia (13). In addition, nine months of supplementation with 1,000 IU of vitamin D3 per day was reported to decrease parathyroid hormone (PTH) levels, while no significant effects were observed on total calcium, action potential protein, calciuria, and s-CTx in the women aged 50-65 years (14).

In addition to pharmaceutical treatments, long-term physical exercise is believed to have beneficial effects on the bone mass (15). Studies investigating the effect of physical exercise on bone health have indicated that exercises such as running and jumping could increase the ground reaction force, thereby influencing osteogenic stimulus more effectively compared to resistance training, which could increase the joint reaction force (16). Several studies have been focused on the effects of physical exercise on bone reabsorption markers. In a research, 12 weeks of hypertrophy exercise was reported to significantly affect bone turnover markers and structure in growing male rats (17). Furthermore, eight weeks of circuit training has been reported to positively affect the bone metabolism markers and bone density in elderly women with osteopenia (18), while 16 weeks of combined physical exercise has been reported to have no significant effect on NTx although it could significantly increase serum ALP in postmenopausal women (19).

Several studies have denoted the beneficial effects of vitamin D supplementation and physical exercise on various diseases. However, further investigations are required considering the conflicting results regarding the effects of vitamin D supplementation and physical exercise on bone resorption markers, while data are also scarce on the interactive effects of endurance training and vitamin D supplementation.

The present study aimed to investigate the effects of endurance training with vitamin D supplementation on the bone resorption markers in the rats exposed to the oxidative damage induced by H_2O_2.

Materials and Methods

This experimental study was conducted on 60 adult Wistar rats (weight: 200±20 grams). The animals were purchased from the Animal Breeding Center at Shiraz University of Medical Sciences in Shiraz, Iran. After transferring the rats to the Animal House of the Physiology Research Center at Kerman University of Medical Sciences in Kerman, Iran, they were kept in animals cages for one week in standard conditions with the temperature of 22±2°C, 12-hour light/dark cycle, and free access to water and rodents' special food (crude protein: 23, crude fat: 3.5-4.5, crude fiber: 4-4.5, ash: maximum of 10, calcium: 0.95-1, phosphorus: 0.65-0.7, salt: 0.5-0.55, humidity: maximum of

10, lysine: 1.15, methionine: 0.33, methionine plus cysteine: 0.63, threonine: 0.72, tryptophan: 0.25), which was purchased from Pars Food Company in Tehran, Iran.

Grouping of the Animals

Before grouping, the animals ran on a treadmill for one week. Afterwards, they were randomly assigned to 10 groups of six, as follows: 1) control, 2) H_2O_2 (1 mmol/kg), 3) H_2O_2 (2 mmol/kg), 4) H_2O_2 (1 mmol/kg) with vitamin D, 5) H_2O_2 (2 mmol/kg) with vitamin D, 6) training with H_2O_2 (1 mmol/kg), 7) training with H_2O_2 (2 mmol/kg), 8) training with H_2O_2 (1 mmol/kg) and vitamin D, 9) training with H_2O_2 (2 mmol/kg) and vitamin D, and 10) sham (dimethyl-sulfide [DOMS]).

H_2O_2 Injection

Groups two, four, six, and eight were administered with 1 mmol/kg of H_2O_2 (15), and groups three, five, seven, and nine were administered with 2 mmol/kg of H_2O_2 (16) via intraperitoneal injection three times per week on coupled days.

Vitamin D Supplementation

Groups four, five, eight, and nine were administered with 0.5 gram/kgμ of vitamin D_3 via intraperitoneal injection daily. It is notable that normal saline was used to reach the appropriate dosage, and DOMS was used to dissolve vitamin D_3 in saline (17).

Endurance Training Protocol

Groups six, seven, eight, and nine performed endurance training on a rodent treadmill for eight weeks, and the slope of the treadmill was set steady at 10 degrees. The speed and duration of the training gradually increased from approximately eight meters per minutes for 30 minutes during the first week to 12 meters per minute for 30 minutes during the second week, 16 meters per minute for 45 minutes during the third week, and 20 meters per minute for 45 minutes in the fourth week. During weeks 5-8, the speed remained stable at 20 meters per minute for 60 minutes (18).

Measurement of the Research Variables

To measure the research variable 24 hours after the last training session, the animals were anesthetized through the inhalation of chloroform, and blood samples were collected. Serum levels of TRACP/5B (Cat No. CK-E30288) and NTx (Cat No. E0010Ra) were measured using the ELISA assay commercial kit (Shanghai Crystal Day Biotech Co., Ltd.). All the animal experiments were conducted in accordance with Helsinki Statement (2008), and the study protocol was approved by the Ethics Committee of the Ministry of Health and Medical Education, Kerman University of Medical Sciences and Health Services (IR.KMU.REC.1396.1562).

Statistical Analysis

Data analysis was performed in SPSS version 21 using the Kolmogorov-Smirnov test, two-way analysis of variance (ANOVA), one-way ANOVA, and Tukey's post-hoc test at the significance level of $P \leq 0.05$.

Results

Table 1 shows the mean values of the research variables in the study groups.

Table 1. Mean Research Variables

Variable Group	TRACP/5 (nmol/ml)	NTx (nmol/ml)
Sham	7.67±1.97	4.26±0.97
Control	9.20±4.45	5.51±0.97
1 mmol/kg of H_2O_2	7.49±1.59	6.12±1.76
1 mmol/kg of H_2O_2 with Vitamin D	6.79±2.59	6.32±2.09
2 mmol/kg of H_2O_2	7.21±2.04	6.42±1.11
2 mmol/kg of H_2O_2 with Vitamin D	7.48±0.98	6.58±1.32
Endurance Training with 2 mmol/kg of H_2O_2	7.24±1.81	7.95±0.98
Endurance Training with 1 mmol/kg of H_2O_2	7.82±1.94	6.65±1.32
Endurance Training with 1 mmol/kg of H_2O_2 and Vitamin D	7.11±1.66	6.34±1.45
Endurance Training with 2 mmol/kg of H_2O_2 and Vitamin D	8.47±2.27	6.86±1.44

TRACP/5B: tartrate-resistant acid phosphatase 5b; NTx: N-telopeptides

Effects of 1 and 2 mmol/kg of H_2O_2 on the Research Variables

The effects of 1 and 2 mmol/kg of H_2O_2 on the research variables were investigated using one-way ANOVA. According to the information in Table 2, no significant difference was observed in the serum level of TRACP/5B ($F= 0.83$; $P=0.48$) between the groups of control, sham, 1 mmol/kg

of H_2O_2, and 2 mmol/kg of H_2O_2. However, a significant difference was observed in the serum NTx level between the groups of control, sham, 1 mmol/kg of H_2O_2, and 2 mmol/kg of H_2O_2 (F=4.66; P=0.009). According to the results of Tukey's post-hoc test, the administration of 1 mmol/kg of H_2O_2 (M=0.60; P=0.76) and 2 mmol/kg of H_2O_2 (M=0.91; P=0.47) had no significant effect on the increased serum level of NTx in the animals. On the other hand, the NTx level was observed to be significantly lower in the animals administered with 1 mmol/kg of H_2O_2 (M=-1.85; P=0.02) and 2 mmol/kg of H_2O_2 (M=-2.15; P=0.009) compared to the sham group.

Table 2. Results of One-way ANOVA for Comparison of Research Variables in Groups of Control, Sham, 1 mmol/kg of H_2O_2, and 2 mmol/kg of H_2O_2

Variable	Factor	Sum of Squares	Mean of Squares	F	P-value
TRACP/5B	Inter-group	19.15	6.38	0.83	0.48
	Intra-group	213.46	7.62		
	Total	232.61			
NTx	Inter-group	21.91	7.30	4.66	0.009
	Intra-group	43.88	1.56		
	Total	65.80			

TRACP/5B: tartrate-resistant acid phosphatase 5b; NTx: N-telopeptides

Effects of Endurance Training and Vitamin D Supplementation on the Research Variables

According to the results of two-way ANOVA (Table 2), eight weeks of endurance training (F=0.21; P=0.65; effect size=0.007) and vitamin D supplementation (F=1.008; P=0.32; effect size=0.03) had no significant effects on the increased level of TRACP/5B in the rats exposed to 1 mmol/kg of H_2O_2. Furthermore, eight weeks of endurance training with vitamin D supplementation had no interactive effect on the increased level of TRACP/5B in the rats exposed to 1 mmol/kg of H_2O_2 (F=0.001; P=0.99, effect size=0.001).

According to the obtained results, eight weeks of endurance training (F=0.21; P=0.64; effect size=0.001) and vitamin D supplementation (F=0.009; P=0.92; effect size=0.001) had no significant effect on the increased level of NTx in the rats exposed to 1 mmol/kg of H_2O_2. In addition, eight weeks of endurance training with vitamin D supplementation had no interactive effect on the increased level of NTx in the rats exposed to 1 mmol/kg of H_2O_2 (F=0.18; P=0.67; effect size=0.006).

According to the results of two-way ANOVA (Table 2), eight weeks of endurance training (F=0.61; P=0.44; effect size=0.02) and vitamin D supplementation (F=1.32; P=0.26; effect size=0.04) had no significant effect on the increased level of TRACP/5B in the rats exposed to 2 mmol/kg of H_2O_2. Moreover, eight weeks of endurance training with vitamin D supplementation had no interactive effect on the increased level of TRACP/5B in the rats exposed to 2 mmol/kg of H_2O_2 (F=0.52; P=0.47; effect size=0.01).

According to the obtained results, eight weeks of endurance training could significantly increase the NTx level in the rats exposed to 2 mmol/kg of H_2O_2 (F=4.30; P=0.04; effect size=0.13), while eight weeks of vitamin D supplementation had no significant effect on the increased level of NTx in the rats exposed to 2 mmol/kg of H_2O_2 (F=1.15; P=0.29; effect size=0.04). In addition, eight weeks of endurance training with vitamin D supplementation had no interactive effect on the increasing of NTx level in the rats exposed to 2 mmol/kg of H_2O_2 (F=2.04; P=0.16; effect size=0.06).

Table 3. Results of Two-way ANOVA for Review of Effects of Endurance Training and Vitamin D Supplementation on Research Variables in Rats Exposed to Oxidative Damage Induced by H_2O_2

Oxidative Damage Induced by H_2O_2	Parameter	Two-way ANOVA								
		Endurance Training			Vitamin D			Interactive Effects of Endurance Training and Vitamin D		
		F	P-value	Effect Size	F	P-value	Effect Size	F	P-value	Effect Size
1 mmol/kg of H_2O_2	TRACP/5B	0.21	0.65	0.007	1.00	0.32	0.03	0.001	0.99	0.001
	NTx	0.21	0.64	0.008	0.009	0.92	0.001	0.18	0.67	0.006
2 mmol/kg of H_2O_2	TRACP/5B	0.61	0.44	0.02	1.32	0.26	0.04	0.52	0.47	0.01
	NTx	4.30	0.04€	0.13	1.15	0.29	0.04	2.04	0.16	0.06

€: significant effect of endurance training on increasing NTx; TRACP/5B: tartrate-resistant acid phosphatase 5b; NTx: N-telopeptides

Discussion

According to the results of the present study, the administration of 1 and 2 mmol/kg of H_2O_2 had no significant effects on the serum levels of NTx and TRACP/5B. It is believed that high levels of ROS could block and reduce osteoblast activity and differentiation. As a result, mineralization and osteogenesis were affected by ROS and H_2O_2 (20, 21), thereby increasing bone remodeling turnover with the subsequent alteration and reduction of the bone mass. Antioxidants exert opposing effects and contribute to the differentiation of osteoblasts and bone formation, thereby maintaining the vital osteocytes that contribute to osteoblast activity and osteogenesis, while reducing osteoclast differentiation and their activity (1). Based on findings of the current research regarding the effects of H_2O_2 on the studied variables, it could be stated that the cell response to hydrogen peroxide may vary, and the influential factors include the concentration of catalase, ability to repair DNA, levels of the radicals forming hydroxyl, and exposure to H_2O_2 (22). On the other hand, exposure of cells to 0.1 and 0.3 micrograms of beryllium for 30 minutes has been reported to reduce ATP (23).

According to the results of the present study, eight weeks of aerobic training increased the serum level of NTx in the rats exposed to 2 mmol/kg of H_2O_2. However, the training intervention had no significant effect on the increasing of the TRACP/5B level in the rats exposed to 1 and 2 mmol/kg of H_2O_2, as well as those exposed to 1 mmol/kg of H_2O_2. Physical exercise is defined as well-structured, planned, and repetitive physical activity, aiming at improving health and wellbeing and maintaining physical fitness (24). It has been well established that physical activity increases the bone mass, reduces calcium secretion, and increases its absorption efficiency. Serum calcium elevation contributes to the saving of the serum levels of vitamin D and NTx reduction (25, 26). On the other hand, physical exercise results in weight loss, fat lipolysis, and the subsequent movement of vitamin D from the adipose tissue, which in turn increases its serum levels (25). As a result, vitamin D could increase the serum concentrations of block PTH and decrease the ratio of RANKL/osteoprotegerin to diminish the bone loss (27).

Several studies have investigated the effect of endurance training on bone metabolism. In line with the current research, the high force of eccentric contractions has been reported to significantly influence osteocalcin and TRACP-5B in young, untrained men (28). On the other hand, long-term, combined physical exercise has been reported to have no significant effect on NTx, while it has been shown to significantly increase serum ALP in postmenopausal women (19). Such discrepancies could be attributed to the differences in the sample populations, exercise intensity, type of exercises, and duration of exercises.

According to the literature, physical exercise and training could prevent osteoporosis in the elderly as a non-medication preventive strategy. The interactions of mechanical loading, hormones or cytokines with signaling pathways induced by physical exercise could enhance bone formation and reduce bone resorption, thereby leading to the maintenance of a healthy skeleton (29).

According to the findings of the current research, eight weeks of vitamin D supplementation had no significant effects on the increasing of TRACP/5B and NTx levels in the rats exposed to 1 and 2 mmol/kg of H_2O_2. Previous studies have also indicated that active vitamin D administration

could decrease the serum concentrations of PTH (30). The signaling of PTH receptors in the osteoblasts and osteocytes could increase the ratio of RANKL/osteoprotegerin (a decoy receptor of RANKL) to enhance the recruitment and activity of the osteoclasts and stimulate bone resorption (31). As such, the inhibitory effects on PTH caused by vitamin D administration could result in higher bone mineral density (27).

Considering that the animals in the present study had a controlled diet and lifestyle in identical, optimal conditions and hydrogen peroxide had no significant effects on the bone metabolic markers, our findings could be justified by the fact that the use of vitamin D led to the increased serum levels of these markers, and the increased levels of vitamin D could improve metabolism and the ability to absorb calcium from foods, which in turn leads to the increased serum calcium levels, as well as bone calcium resorption (32). However, the proper interpretation of our findings requires further information and measurement of more markers (e.g., bone density), bone-specific ALP, and tissue and serum levels of calcitonin.

Some of the studies in this regard have investigated the effects of vitamin D on the bone metabolism markers. For instance, Nakamura et al. reported that vitamin D supplementation had a significant effect on TRACP-5b, while it decreased NTx, and improved the ALP levels and bone density in patients with osteoporosis (30). Other similar studies have also demonstrated the effects of vitamin D on the bone metabolism markers in patients with osteoporosis (13) and osteopenia (14). However, the mentioned findings are inconsistent with the results of the present study, and this discrepancy could be due to the differences in the sample populations and administered vitamin D concentrations. Considering that H_2O_2 did not alter the metabolic markers in the current research, vitamin D seems to have better application in the animal models of osteoporosis or in cases with vitamin D deficiency.

According to the findings of the current research, eight weeks of aerobic training with vitamin D supplementation had no interactive effect on the increasing of the TRACP/5B and NTx levels in the rats exposed to 1 and 2 mmol/kg of H_2O_2. Previous studies in this regard have discussed the role of physical exercise and vitamin D supplementation separately on bone health. Based on the limited studies on the effects of vitamin D and exercise on bone health, it seems that these interventions could exert interactive effects on the increasing of the serum levels of vitamin D and calcium resorption through the simultaneous improvement of the overall metabolism in the body, decreasing the body fat mass, and increasing the lipolysis of the adipose tissue (25-27).

According to the literature, physical exercise combined with vitamin D supplementation has interactive effects on the reduction of parathyroid hormones and elevation of ALP in postmenopausal women (33), as well as the reduction of inflammation and fat mass in non-athlete men (34). According to the results of the present study, the effects of physical exercise and vitamin D supplementation at various concentrations of H_2O_2 varied in terms of the changes in the serum levels of vitamin D and calcium. It is notable that these findings may depend on the concentration of H_2O_2 and variable effects of its levels.

No prior studies were found regarding the interactive effects of physical exercise and vitamin D on the levels of vitamin D, calcium, TRACP/5B, and NTx. The main strength of the current research was the review of the effects of two concentrations of H_2O_2 on TRACP/5B and NTx. On the other hand, one of the limitations of the present study was the lack of measuring food intake, calorie intake, and protein intake. Therefore, it is recommended that further investigations be conducted in this regard. Considering the key role of markers such as bone-specific ALP, calcitonin, parathyroid hormone, and bone density, no measurement of these variables in the current research was another limitation. It is suggested that further investigations in this regard be focused on the measurement of these markers for the accurate interpretation of our findings.

Conclusion

According to the results, endurance training could significantly increase the NTx serum level in the rats exposed to the oxidative damage induced by 2 mg/kg of H_2O_2. Therefore, it could be concluded that in the conditions with the oxidative damage induced by H_2O_2, endurance trainings could be used to improve the bone resorption markers.

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