

## Vitamin D and Training Modulates Gene Expression of TLR4 and NFkB in Lung Tissue of Obese Rats in an Experimental Study

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### Abstract

**Objective:** Physical exercise has different effects on oxidative stress. Oxidative stress influences TLR4 and NFkB gene expression. The purpose of this study was to investigate the effect of aerobic training and vitamin D on gene expression of TLR4 and NFkB in lung tissue of obese rats exposed to oxygenated water.

**Materials and Methods:** In an experimental study, 30 obese male wistar rats were randomly divided into five groups: control, oxygenated water, oxygenated water + vitamin D, oxygenated water + aerobic training, and oxygenated water + aerobic training + vitamin D. All the rats were injected intraperitoneally with oxygenated water. Vitamin D was performed by intraperitoneal injection of 0.5 µg daily for eight weeks. The aerobic training protocol included 8 weeks, 5 sessions per week running on treadmill. TLR4 and NFkB gene expression of lung tissue were investigated using real time & PCR. Two-way ANOVA and Bonferroni post hoc test were used to analysis the data. The significant level was set at  $P$ -value < 0.05.

**Results:** Aerobic training significantly reduced TLR4 expression compared with other groups ( $P$ -value: 0.046) but did not significantly affect the expression of NFkB gene ( $P$ -value: 0.261). Vitamin D alone and aerobic training and vitamin D interaction did not significantly alter the gene expression of TLR4 ( $P$ -value: 0.072 and  $P$ -value: 0.695, respectively) and NFkB ( $P$ -value: 0.243 and  $P$ -value: < 0.195, respectively).

**Conclusion:** It seems that performing aerobic training is likely to be beneficial in reducing oxidative stress and inflammation compared to inactivity.

**Keywords:** Aerobic training, Oxidative stress, TLR4, NFkB

### Introduction

As one of the vital respiratory organs in humans, the lungs play an important role in the exchange of gases, fluid excretion and exchange, acid-base balance,

and other biological processes. Lung tissue is exposed to the adverse effects of the external environment, which increases the lung capacity against oxidative damage. (1).

The reactive oxygen species (ROS) are associated with various diseases, such as diabetes, heart, liver, and kidney diseases (2).  $H_2O_2$  is a mild and relatively stable oxidant widely used as a ROS marker to evaluate the response of cells to oxidative stress(3), because it can penetrate cells and can easily pass through biological membranes, causing oxidative stress(4).

Toll-like receptor 4 (TLR4) identifies pathogen-related molecular patterns and plays a critical role in lung injury (2, 5). Evidence has shown that TLR4 stimulated by LPS causes lung injury by activating the TLR4/nuclear factor kappa B (NFkB) pathway, which is associated with inflammatory response (6,7).

The NFkB is located in the downstream TLR4 signaling pathway and plays a pivotal role in regulating immune response, cell proliferation and cell differentiation(8). NFkB transcription factor is involved in regulating the expression of cytokines and other mediators that are involved in acute inflammatory responses associated with ROS overproduction (9). Many reports have shown that a wide variety of antioxidants inhibit NFkB activity (10,11).

Vitamin D as an antioxidant has been demonstrated to reduce inflammation by inhibiting TLR4 expression (12,13). Sport exercises, especially aerobic, improve cardiovascular and respiratory fitness (14). Aerobic training temporarily increases the production of ROS, but reduces the diseases associated with oxidative stress and long-term aerobic training protects the body against oxidative stress. Physical exercises regulate the antioxidant defense mechanisms and regenerate proteins in the body through redox-sensitive transcription factors, including NFkB (15). The temporary increase in ROS levels activates the NFkB signaling cascade, which in the long term upregulates defense antioxidant mechanisms to counteract and prevent the formation of defective inflammatory cycles and oxidative stress (15). Therefore, considering the dual effects of physical exercise on oxidative stress, the role

of vitamin D3 as an antioxidant, and on the other hand, the lack of study on the interaction effect of aerobic training and vitamin D3 on TLR4 and NFkB gene expression in lung tissue, the present study investigated the combination effects of aerobic training and vitamin D on the expression of TLR4 and NFkB genes in the lung tissue of rats exposed to oxygenated water.

## Materials and Methods

In an experimental study, 30 obese male Wistar rats and aged 8-10 weeks were procured from Shiraz University Animal Center. All rats were kept in polycarbonate cages (5 mice per cage) at  $22 \pm 2$  °C, 55% humidity and 12-12 h of light/dark cycles, with ad libitum access to water and food. All animal experiments were performed according to the ethical guidelines and permission of the research deputy of Kerman University of Medical Sciences. The rats were allowed two weeks to acclimatize to the environment before the start of the protocol and then randomly divided into five groups: control, oxygenated water, oxygenated water+ vitamin D, oxygenated water+ aerobic training, and oxygenated water+ aerobic training +vitamin D. Aerobic training groups were allowed 10 days to acclimatize to treadmill.

All rats were intraperitoneally injected with 0.1 mg/kg body weight of oxygenated water (Merck, Germany) three times a week on Saturdays, Mondays, and Wednesdays (16,17). Rats that were assigned to undergo aerobic training ran on treadmill daily for 8 weeks (Figure 3.1). Rats practiced treadmill in the first week at a speed of 8 m/min and 10 degree steep for 30 min, in the second week, at 12 m/min and the same steep for the same duration, in the third week at 16 m/min and the same steep for 45 min, and in the fourth week at 20 m/min and the same steep for 45 min. During the fifth to eighth weeks, rats were practiced at 20 m/min and 10-degree steep for 60 min per day (14).

Vitamin D3 (DITHRECOL, Caspian Co.) at a concentration of 300000 UI/ml was

intraperitoneally injected to the rats that were decided to be treated with vitamin D. Normal saline was used for dilution to obtain the appropriate dose, and dimethyl sulfoxide (DMSO) was used to dissolve vitamin D3 in saline. Treatment with vitamin D3 in vitamin D3 exposed groups was performed by intraperitoneal injection of 0.5 µg vitamin D3 daily for eight weeks (18).

Twenty four hours after the last session of the protocol and 12 hours of fasting, the rats were anesthetized by inhalation of chlorophyll and then sacrificed. The lungs were carefully taken out and immediately immersed in liquid nitrogen and stored at 75 °C until subsequent experiments. Then, various steps were performed according to the instructions of the RNA extraction kit until the final stage of extraction and pure RNA preparation were accomplished.

The extracted RNA solution was cleansed with the DNaseI enzyme (Synagena) from any DNA contamination and RNA degradation enzymes. The optical absorbance at 280-260 nm wavelength was obtained 1.8-2 by spectrophotometer for all extracted samples. Then, the quality of the extracted RNA was investigated by electrophoresis using 1% agarose gel.

For synthesis of cDNA, 5 µg of each of the mRNA samples, Oligo-dT primers (Parstous Biotechnology) and reverse transcription enzyme were used according to the instructions of a cDNA synthesis kit (Parstous Biotechnology). Primers were designed by using Primer 3 software.

The relative expression level of TLR4 and NFκB genes were determined by real time PCR. Reaction solution contained 1 µl of each primer, 1 µl of cDNA, 12 µl Cyber Green and 6 µl of distilled water without nucleic acid. The PCR conditions included an initial denaturation at 95 °C for 5 min, followed by 45 cycles at 95 °C for 20 s and 60 °C for 1 min. The relative expression level of TLR4 and NFκB in the lung tissues was determined by the  $2^{-\Delta\Delta C_t}$  method and using beta-actin as the internal control gene.

### Statistical analysis

All data were expressed as mean and standard deviation. Two-way analysis of variance was used to determine the effects of exercise and vitamin D alone and the interaction effect of exercise and vitamin D on the studied outcomes in each group. If a difference was observed, Bonferroni's post-hoc test was used. The significance level (*P*) was considered < 0.05 for all calculations. All statistical analyses were performed using the SPSS version 24.

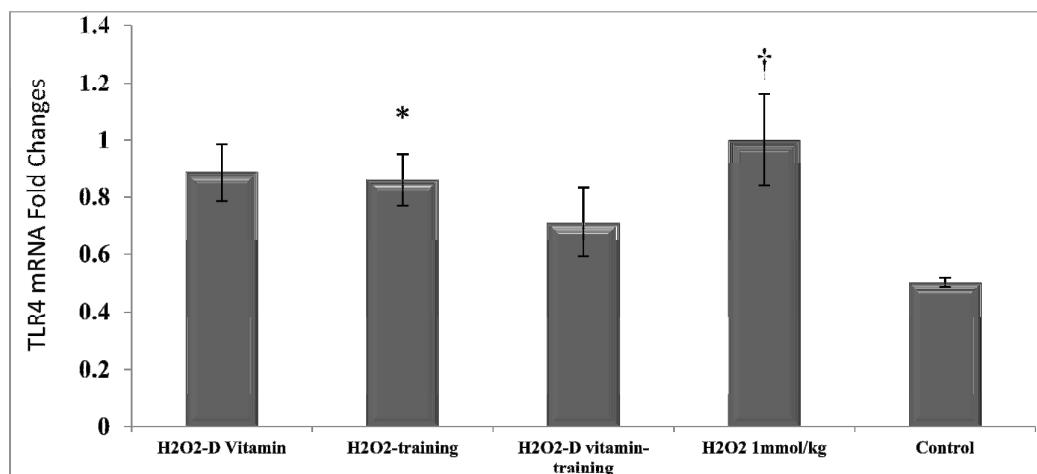
### Ethical considerations

This study was approved by the ethics committee of Kerman University of Medical Sciences and Health Services Research and Technology Center and the Ministry of Health and Medical Education. (code IR.KMU.REC.1396.1562).

### Results

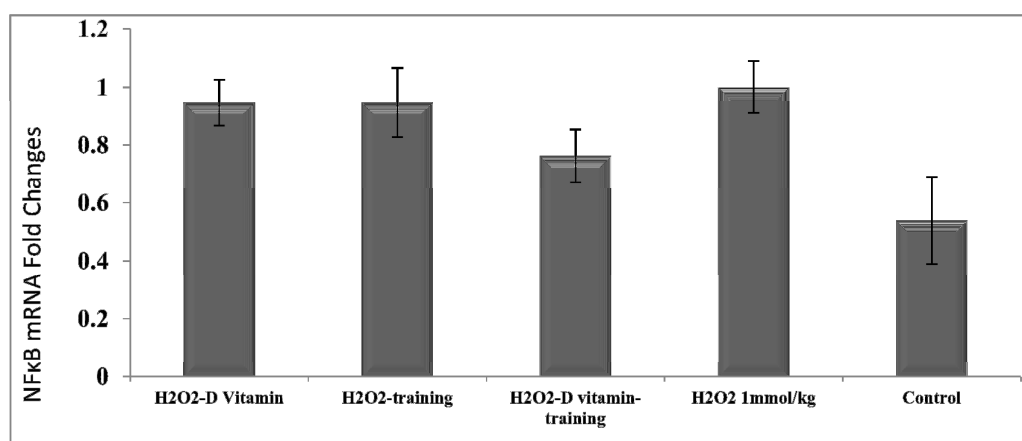
The induction of H<sub>2</sub>O<sub>2</sub> 1mmol/kg significantly increases gene expression of TLR-4 (*P*-value= 0.001). Aerobic training caused a significant reduction in the expression of the TLR4 gene in comparison with oxygenated water group (*P*-value= 0.046). However, vitamin D had no significant effect on TLR-4 expression compared to oxygenated water group (*P*-value= 0.072). Training and vitamin D interaction effect did not have a significant effect on TLR4 expression in comparison with oxygenated water group (*P*-value= 0.695). (Figure 1)

The induction of H<sub>2</sub>O<sub>2</sub> 1mmol/kg significantly increases gene expression of NFκB (*P*-value= 0.001). Aerobic training had no significant effect on NFκB gene expression in comparison with oxygenated water group (*P*-value= 0.261). Vitamin D also did not have a significant effect on NFκB gene expression compared to oxygenated water group (*P*-value= 0.243). Also, the interaction effect of training and vitamin D on NFκB gene expression was not significant in comparison with oxygenated water group (*P*-value= 0.195) (Figure 2).



**Figure 1. TLR-4 gene expression in the studied groups.**

Data are expressed as mean (± standard deviation). \* significant reduction to H2O2 group ( $P$ -value= 0.001). † significant elevation to control group ( $P$ -value= 0.001).



**Figure 2. NFκB gene expression in the studied groups. Information is reported based on average and standard deviation.**

## Discussion

Many studies have shown that oxidative stress is involved in the activity of TLR4 and NFκB (19, 20). In the present study, we investigated the combination effect of 8 weeks aerobic training and vitamin D on the expression of TLR4 and NFκB genes in the lung tissue of obese rats exposed to oxygenated water. The results of real time PCR showed that TLR4 significantly decreased in aerobic training group compared to other groups, but vitamin D had no significant effect on TLR4 gene expression compared to other groups. Besides that, the interaction effect of aerobic training

and vitamin D was not significant in comparison with other groups. Regarding NFκB, aerobic training and vitamin D alone and even their combination did not significantly change the gene expression. Regarding the role of oxidative stress in inflammation, many studies have been conducted on the effects of chronic induction of exogenous H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> induction is considered one of the models of oxidative stress induction. Reactive oxygen species (ROS) such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) are produced during normal cell metabolism and play an important role in signaling pathways. H<sub>2</sub>O<sub>2</sub> also produces toxic effects because it can

produce new radicals and cause damage to the main constituents of the cell. In addition, it has been shown that exogenous  $H_2O_2$  causes an imbalance between the production and elimination of ROS compared to the pre-oxidative conditions that is often referred to as oxidative stress (21). Because  $H_2O_2$  can be released easily through a cell membrane of any cell diameter size due to its solubility in both lipid and hydrous environments and its relatively low reactivity before it reacts with certain molecular targets.  $H_2O_2$  can regulate essential cell functions such as cell growth, proliferation, and differentiation, or can cause cell death through apoptosis or necrosis(22).

Although previous studies reported a reduction in the expression of TLR4 gene and serum NF $\kappa$ B levels after exercise(23,24), no study, as far as we searched, has yet been conducted on the effect of aerobic training and vitamin D on the expression of the TLR4 and TFL $\alpha$  genes in the lung tissues exposed to oxygenated water, so that we cannot compare our results with other evidence. However, both variables are the consequences of oxidative stress and contribute to the development of inflammation and oxidative stress in lung tissue.

Our results showed a significant reduction in the expression of TLR4 in the aerobic training group compared to other groups and an insignificant reduction in the expression of NF $\kappa$ B gene expression in the aerobic training group compared to other groups. The precise mechanism of reduced expression of TLR4 by exercise has not yet been well known, but it has been attributed to various pathways. For example, decreased expression of TLR4 due to aerobic training may be due to less exposure to endogenous ligands such as LPS, peptidoglycans, and heat shock proteins, all of which may increase during each exercise trial throughout the training program and cause reduction in the expression of TLR4 (25). In addition, recent studies have shown that acute exercises lead to changes in the expression of anti-inflammatory genes that regulate the expression and function of TLR4 (26).

High glucose levels in vitro (laboratory environment) and hyperglycemia in vivo (in the body) have been found to be associated with increased TLR4 expression, and decreased plasma glucose by exercise may be a potential mechanism for reduced expression of TLR4 protein. Reduced plasma glucose concentrations by exercise provides evidence for improving glucose control, which is likely to contribute to the reduction in TLR4 (25). Evidence suggests that the TLR4/NF $\kappa$ B signaling pathway plays an important role in the pathogenesis of many human diseases, including pulmonary diseases. In a study with murine mouse model of fetal, oxidative-induced injury, high levels of oxygen were used for oxidant induction and hypoxia. Their observations showed that prolonged exposure to oxidants could lead to respiratory failure and death (27). Also, ROS can activate the signaling pathway that leads to the NF $\kappa$ B activation. Special intracellular events are influenced by oxidative stress that have not yet been fully understood. Certain kinases such as SKY, AKT and the IKK complex are activated by  $H_2O_2$  (28). Evidence obtained on the relationship between ROS and NF $\kappa$ B has shown that NF $\kappa$ B nuclear replacement can be increased by exposure to ROS, and cytokines-induced NF $\kappa$ B activation can be prevented by antioxidants (10,11).

Vitamin D, as an antioxidant, also plays an important role in the regulation of the immune/inflammatory system by regulating the production of inflammatory cytokines and inhibiting the proliferation of anti-inflammatory cells, both of which play a role in the pathogenesis of inflammatory diseases (29,30). The chronic activation of NF $\kappa$ B is associated with different pathological conditions. Inhibition of NF $\kappa$ B activation is an effective strategy to prevent the production of inflammatory and proinflammatory cytokines. In the present study, the insignificant reduction of NF $\kappa$ B due to exercise can be attributed to the beneficial effects of exercise in reducing the expression of the NF $\kappa$ B gene. The insignificant reduction in the expression of the



gene may be attributed to the reduction of TLR4 expression and oxidative stress, but the change in the intensity and duration of the exercise could have produced more pronounced effects. The results also showed an insignificant reduction in TLR4 expression and an insignificant increase in NF $\kappa$ B by vitamin D, and probably vitamin D could bind to TLR4 and inhibit its expression (8). Vitamin D, as an antioxidant, can reduce oxidative stress by inducing cytokine production by LPS (31). Vitamin D inhibits NF $\kappa$ B activity and reduces KPNA4 levels. The NF- $\kappa$ B activated via KPNA4 is transported from the cytoplasm to the nucleus. Vitamin D inhibits the expression of KPNA4 through enhancing levels of vitamin D receptors or an upregulation of protein expression. This vitamin D-dependent process suppresses TNF- $\alpha$  induction, NF $\kappa$ B nuclear transfection, and transcriptional activity (30). Overall, it is likely that changing the intensity of exercise and increasing the dose of vitamin D, respectively, induced inhibitory effects on NF $\kappa$ B gene expression and significant decrease in TLR4 and NF $\kappa$ B.

## Conclusions

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In summary, aerobic training reduced the TLR4 gene expression significantly, but did not have a significant effect on NF $\kappa$ B gene expression. Vitamin D did not have a significant effect on the gene expression of the TLR4 and NF $\kappa$ B. No significant interaction effect of aerobic training and vitamin D was observed on the gene expression of the TLR4 and NF $\kappa$ B. In general, aerobic training can be beneficial, in comparison with physical inactivity, to reduce the oxidative stress and subsequent inflammation.

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## Conflict of Interest

The authors declare that they have no conflicts of interest.

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