# The evaluation of the effect of periodic resistance training along with vitamin $D_3$ consumption and mesenchymal stem cell transplantation on cortical TNF-β level in streptozotocin-induced diabetic rats

**Running title:** Resistance training, vitamin D3 consumption, and stem cell transplantation in diabetic rats

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**Abstract**

**Background:** Inflammation plays a major role in the development and progression of diabetes. Vitamin D deficiency and physical inactivity can also increase the risk of developing type 2 diabetes. Combined therapeutic strategies are promising approaches for the treatment and prevention of diabetes. This study aimed to investigate the effects of resistance training, vitamin D3 supplementation, and adipose-derived mesenchymal stem cell (MSC) transplantation on tumor necrosis factor-beta levels in the cerebral cortex of diabetic rats.

**Methods:** Eighty male Wistar rats (weighing 290±19 g) were randomly divided into 10 groups: healthy control, sham, diabetes, training, vitamin D, MSC, training+vitamin D, MSC+training, MSC+vitamin D, and training+MSC+vitamin D. Training groups were subjected to a resistance training program on a ladder. MSC groups received $1.5 \times 10^6$ MSCs, and vitamin D supplementation groups received 1 microgram/kilogram vitamin D3 eight times. Cortical TNF-β levels and fasting serum glucose levels were measured.

**Results:** After 6 weeks, the combination of resistance training with vitamin D3 supplementation and MSC transplantation (P=0.018) as well as the combination of resistance training with MSCs (P=0.024) significantly reduced diabetes-induced elevation of TNF-β level.

**Conclusion:** Resistance training with appropriate intensity, duration, and recovery between exercise sessions, combined with MSC transplantation and vitamin D3 supplementation, has profound anti-inflammatory effects on the cerebral cortex tissue of diabetic rats. This type of intervention, especially the transplantation of MSCs, may be a promising protective strategy against some complications of diabetes.

**Keywords:** Resistance training, Vitamin D, Stem cell, Diabetes, Inflammation

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## Introduction

Diabetes is an important global health problem, with a rising prevalence (1). Chronic low-grade systemic inflammation is a well-known hallmark of diabetes (2). Systemic inflammation plays an important role in the pathogenesis of type 2 diabetes mellitus (T2DM) and the development of insulin resistance. The increase in serum levels of inflammatory mediators such as cytokines and C-reactive protein (CRP) in diabetic patients has been well-demonstrated (3). This leads to the development and progression of cardiovascular complications of diabetes and results in tissue damage and impaired wound healing, which in turn increases the susceptibility of diabetics to opportunistic infections (4). At the molecular level, exposure of cells to tumor necrosis factor (TNF)-α, interleukin (IL)-1b, IL-6, or high levels of free fatty acids induces inhibitory phosphorylation of serine residues of insulin receptors, which directly affects insulin resistance (5).

The immunomodulatory effects of regular exercise, particularly resistance training, may also have positive effects on innate immunity, which may be beneficial for the improvement of diabetes profile as well as strength and functional abilities (6-11). Such effects are thought to be dependent on muscle activity and contractility through the production of IL-6, a cytokine that exerts inhibitory effects on several pro-inflammatory cytokines, including TNF-α (12, 13). Resistance training specifically develops total glucose uptake capacity by increasing muscle mass and improving systemic inflammation (14). However, the exact effects of exercise training on glucose metabolism (15) or systemic inflammation (16) are not clear.

Recently, vitamin D has attracted a lot of attention as an important factor that can improve insulin resistance (17), diabetes (18), inflammatory responses, and blood sugar (19, 20). In the last decade, an increasing body of evidence from large-scale observational studies has shown an association between low levels of 25-hydroxyvitamin D and an increased risk of developing T2DM (21), making vitamin D supplementation a potential preventive approach (22). In this regard, the association between low vitamin D levels and impaired insulin secretion, and increased insulin resistance has led to the hypothesis that vitamin D supplementation may reduce the risk of developing T2DM (23, 24).

In addition to physical activity- and nutritional-based interventions, such as vitamin D supplementation, stem cell injection has been proposed as an effective method for the treatment of diabetes (25). Mesenchymal stem cells (MSCs) are beneficial agents for autoimmune and inflammatory diseases (26). In general, modulation of autoimmunity and chronic inflammation is considered a key target of MSC-based therapies (27). Recent studies indicate that MSCs may be able to ameliorate experimental autoimmune encephalomyelitis, the murine counterpart of human multiple sclerosis (28-31). However, the therapeutic potential of MSCs in type 1 diabetes remains largely unknown, as recently reviewed by Abdi et al. (32).

Data regarding the effects of combined resistance training and vitamin D supplementation on inflammatory and glycemic markers of individuals with diabetes and vitamin D deficiency are insufficient. We hypothesized that resistance training, along with vitamin D consumption and MSC injection, can have a synergistic positive effect on patients with T2DM. Therefore, this study was conducted to investigate the effects of 6 weeks of vitamin D intake along with progressive resistance training and MSC injection on the expression of the TNF-β gene in diabetic rats.

## Methods

*Animals*

This study was performed on 80 adult, male, Wistar rats aged 12 weeks (weighing 290 ± 19 g). The animals were caged under 12:12 light: dark cycles and controlled temperature (22 ± 2 °C). They also had access to standard food and water ad libitum. After being familiarized with their living conditions for a week, the animals were randomly assigned to one of the following 10

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groups each containing 8 rats: 1) healthy control, 2) sham, 3) diabetes, 4) diabetes + training, 5) diabetes + vitamin D supplementation, 6) diabetes + MSC injection, 7) diabetes+training+vitamin D supplementation, 8) diabetes+MSC injection+training, 9) diabetes+MSC injection+vitamin D supplementation, and 10) diabetes+training+MSC injection+vitamin D supplementation. The training groups were subjected to a resistance training program on a ladder. The groups received $1.5 \times 10^6$MSCs. The vitamin supplementation groups received 1 microgram/kilogram of vitamin $D_3$ 8 times. The sham group received saline to control the stress caused by the cell and vitamin D injection.
The study protocol was approved by the Ethics Committee of Islamic Azad University, Sari Branch (ethical code: IR.IAU.SARI.REC.1398.123).

*Induction of diabetes*
Diabetes was induced by intraperitoneal injection of streptozotocin (STZ, Sigma Aldrich, USA) at a dose of 60 mg/kg (33). For this purpose, STZ (20 mg/ml) was dissolved in cold 0.1 M citrate buffer (pH 4.5). Non-diabetic rats were injected with the same volume of citrate buffer. Seventy-two hours after the STZ injection and after overnight food deprivation, blood glucose was measured using tail vein blood samples. A blood glucose level greater than 250 mg/dl indicates the successful induction of diabetes.

*Resistance training program*
Resistance training was performed using a 76 cm ladder with a slope of 80 degrees and a width of 19 cm (34). There were 47 steps across the ladder. Before the induction of diabetes, the rats were trained to climb the ladder from the bottom to the top. For this purpose, they were placed at the bottom of the climbing training equipment and were motivated to climb the ladder by touch and manual stimulation. Once the rats reached the top of the ladder, they were allowed to rest in a simulated house. Eight days after the STZ injection, the resistance training program was initiated by attaching weights to the base of the tail with adhesive tape and clips. All animals were weighed every 4 days to monitor weight gain, and for the animals in the training groups, the amount of weight to be added to their tails was determined for the rest of the week. Warm-up and cool-down were done with two repetitions, without adding weight to the tail, in the beginning and at the end of each session, respectively.
In the first week, the amount of weights tied to the rats' tails was 50% of the maximum repetition of each animal, which was calculated the day before the start of the resistance training protocol. This amount increased by 10% each week until it reached 100% in the final week. Each session consisted of 3 sets with 5 repetitions, with a one-minute rest between each set (34).

*Vitamin D3 preparation and supplementation*
Vitamin D3 was obtained from the Cayman Chemical and Pharmaceutical Company, Germany. To prepare the supplement, first, 100 µg of calcitriol was dissolved in 1 ml of propylene glycol and then further dissolved in 9% saline. Dihydroxy vitamin D3 (1 µg/kg of body weight) was intraperitoneally injected into the rats, twice a week for the first 2 weeks, and once every weekend for 4 weeks (a total of eight injections) (35).

*Injection of MSCs*
Adipose tissue was used to prepare stem cells. The tissues were washed with phosphate buffer saline (PBS) containing antibiotics (penicillin-streptomycin) four times to remove blood from the solution. Then, the tissues were digested with collagenase-1 for 90 minutes at 37 ºC to separate the cells. Enzyme activity and cell plaque were obtained. Red blood cells were

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removed with lysis buffer. The obtained cells were cultured in a special cell culture flask, counted, and finally prepared for injection (36).
To determine the phenotypic characteristics of the adipose tissue-derived stem cells, the level of CD29 and CD90 expression in these cells was evaluated (37). After induction of anesthesia by intraperitoneal injection of a mixture of 10% ketamine (50 mg/kg) and 2% xylazine (10 mg/kg), the tail of the rats was placed in warm water for 1 minute to dilate and expose the caudal vein. After washing the cells with PBS, about $1.5\times10^6$ stem cells were injected into the caudal vein using an insulin syringe.

*Anesthesia and sampling*
Forty-eight hours after the last training session and after a 12-hour fast, the rats were sacrificed by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (3-5 mg/kg). The animal's head was separated from the body, and the whole brain was removed from the skull (38). The cerebral cortex was separated and immediately placed in liquid nitrogen. After freezing, the tissue was stored at -80 ºC. After homogenization and centrifugation, the level of inflammatory markers was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (HANGHOU Co., China), with a sensitivity coefficient of 195% pg. Serum samples were kept at -80°C until analysis.

*Biochemical measurements*
Serum glucose was measured using an enzymatic colorimetric method (GODPAP, glucose oxidase-amino antipyrine, Pars Azmoun Co., Iran). Frozen tissues were placed in a mortar filled with liquid nitrogen and pounded while floating. The obtained powder (100 mg) was quickly transferred to a microtube and mixed with 1 mL PBS. According to the instructions of the kits, the solution was centrifuged for 5 minutes at 5,000 rpm and a temperature of 2-8 ºC. The supernatant was transferred to another container and stored at -70 ºC for the ELISA test. First, a dilution was prepared from the standard solution according to the kit's instructions. Then, 100 µl of the tissue sample was incubated in wells of a 96-well plate for 2 hours at 37 °C. After 2 hours, the contents of each well were discarded. Next, 100 µl of the primary antibody was added to each well and incubated for 1 hour at 37°C. The contents of each well were discarded, and the wells were washed three times with washing solution. Then, 100 µl of horseradish peroxidase was added to each well and incubated for 1 hour at 37°C. After the contents of each well were discarded and washed five times, 90 µl of 3,3',5,5'-tetramethylbenzidine was added to each well as substrate. The plate was incubated at 37 °C in the dark for 15-30 minutes. Then the enzymatic reaction was stopped by adding 50 µl of stop solution, which created a yellow color. Finally, the absorbance of each sample at 540 nm was read using an ELISA reader. The TNF-β amount was measured by the laboratory kit of HANGHOU company, made in China, with a sensitivity coefficient of %195 pg using the ELISA method.

*Statistical analysis*
All data were expressed as mean ± standard deviation. Statistical analysis of data was carried out in SPSS software (version 22). The normality of data distribution was assessed using the Kolmogorov-Smirnov test. Comparisons were made using one-way analysis of variance (ANOVA) and the least significant difference (LSD) test. The statistical significance level was also set to 0.05.

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## Results

All the rats in the training groups completed the 6-week resistance training program. As shown in Table 1, the rats in the diabetic groups had higher serum glucose levels compared with those in the control and sham groups.

**Table 1.** Mean level of blood sugar in different study groups

| Group | Mean | Standard deviation |
|---|---|---|
| Healthy control | 113.28 | 4.42 |
| Sham | 114.42 | 4.23 |
| Diabetes | 334.71 | 11.48 |
| Diabetes + Training | 293.28 | 11.58 |
| Diabetes + Vitamin D | 334.14 | 7.26 |
| Diabetes + Stem cells | 334.85 | 4.70 |
| Diabetes + Training + Vitamin D | 335.28 | 7.65 |
| Diabetes + Stem cell + Training | 336.42 | 6.52 |
| Diabetes + Stem cell + Vitamin D | 334.42 | 8.69 |
| Diabetes + Training + Stem cell + Vitamin D | 331.85 | 8.39 |

Based on the results of one-way ANOVA, the TNF-β level in the cerebral cortex of rats differed significantly between study groups ($F_{9,79}$=15.17, P=0.005). The LSD post-hoc test showed that TNF-β level differed significantly between the diabetes group and the diabetes+training+vitamin D+MSC group (P=0.018). Moreover, there was a significant difference between the diabetes group and the diabetes+training+MSC group (P=0.024). No significant difference was found between the other study groups in terms of TNF-β expression (Figure 1).

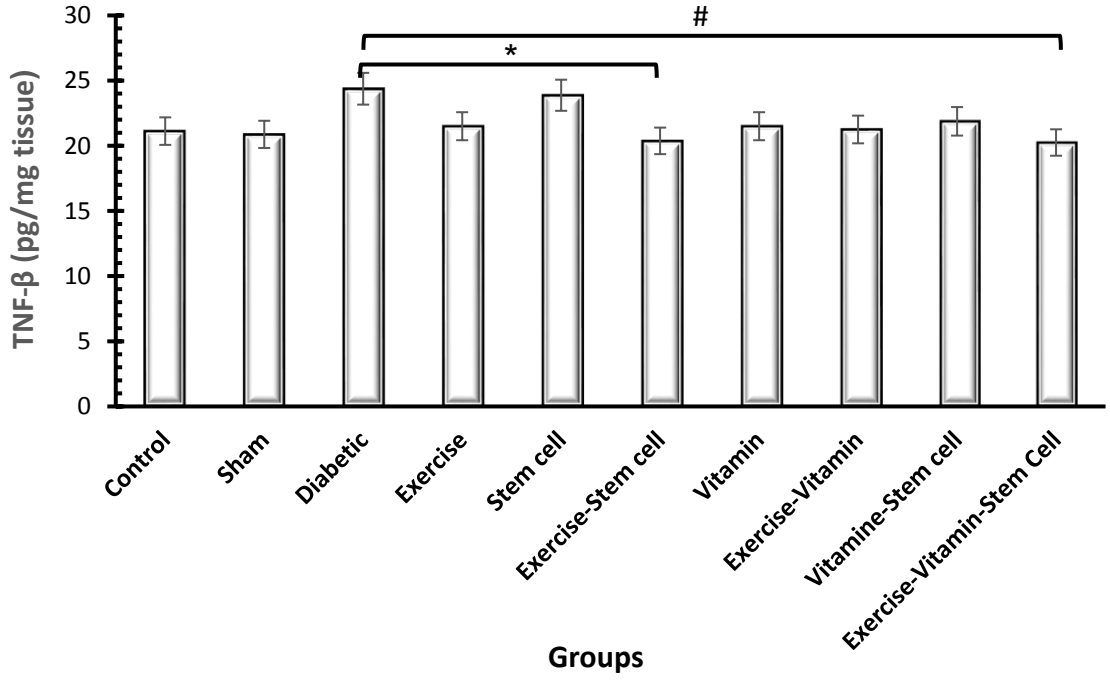


**Figure 1.** Comparison of *TNF-β* levels in the cerebral cortex of diabetic rats. * Indicates a significant difference between the training + stem cells group and the diabetes group. # indicates a significant difference between the training + stem cells + vitamin D group and the diabetes group.

**Discussion**

In this study, 6 weeks of resistance training accompanied by MSC injection and vitamin D3 supplementation decreased TNF-β levels in the cerebral cortex of diabetic rats. However, TNF-β level also decreased significantly in diabetic rats that were subjected to resistance training and MSC injection, indicating that MSC injection with resistance training was a stronger stimulus for decreased TNF-β level. Although the greatest reduction was observed in the combined exercise + vitamin D + MSC group.

Many studies have shown that pro-inflammatory cytokines play an important role in muscle atrophy and physical functional impairment under pathological conditions and aging (39). Inhibiting proinflammatory signaling pathways appears to be a promising strategy to protect against insulin resistance, obesity, and other metabolic syndrome-associated problems, such as cardiovascular disease (40-42). Several studies have shown that physical activity increases serum levels of anti-inflammatory cytokines (43, 44). Based on the anti-inflammatory effects of different physical activities, especially resistance training (45), this type of intervention can be used to control low-grade systemic inflammation in diabetic subjects (46). In addition, resistance training can lower skeletal muscle atrophy in diabetic rats by reducing oxidative stress and inhibiting muscle RING-finger protein-1 expression at both mRNA and protein levels (47). However, it should be noted that inappropriate training intensity can contribute to disease progression and exacerbate the metabolic syndrome-associated metabolic, inflammatory, and stress state (48). In addition, resistance training involving extraverted movements causes greater muscle damage compared with introverted movements (49).

Several studies have shown that muscle damage caused by eccentric exercise induces inflammatory responses characterized by the release of leukocytes and cytokines (50). Cytokine response may vary depending on exercise type, intensity, duration, recovery between exercise bouts, and training status (45, 46). In the present study, using the ladder-climbing training model, the resistance training exercise could significantly reduce TNF-β level in the cerebral cortex of diabetic rats. However, given the short duration of the training program, resistance training alone might not be a strong stimulus for the TNF-β reduction. Nevertheless, resistance training along with MSC injection was able to cause a 15% reduction in TNF-β compared with diabetic rats. In general, MCSs are capable of modulating T-cell responses and/or creating a local immunosuppressive state, making them attractive potential therapeutic agents for the treatment of various chronic inflammatory diseases and immune disorders (36, 51). Comparison of our findings with other studies was limited since the present study was the first to simultaneously investigate the effects of resistance training, vitamin D3 supplementation, and MSC transplantation.

Stem cells are one of the newest treatment strategies that are currently exploited for the treatment of neurological disorders in animal models because these cells have a high self-renewal potential (31, 32). Adipose-derived MSCs are obtained from the subcutaneous adipose tissue and have better cultivation, reproduction, and nerve regeneration capabilities compared with the cells obtained from deeper fat layers (52). Injection of adipose tissue-derived stem cells can be effective in controlling blood sugar and tissue damage caused by diabetes (52). In 2010, Gracia et al. reported that injection of $1\times10^6$ stem cells isolated from human adipose tissue into rats with T2DM could reduce blood sugar (53). In line with our findings, Hashemvarzi demonstrated that stem cell transplantation could regenerate and repair damaged brain tissue (54). Similarly, Ren et al. showed that the level of proinflammatory cytokines, such as TNF-α, in the 24- and 72-hour recovery periods after spinal cord injury in animal models that received MSCs reduces significantly (55), which is in line with the results of the present study. MSCs inhibit the release of proinflammatory cytokines by inducing environmental tolerance and migrating to damaged tissues (56). This may be due to the increased expression of tumor necrosis factor-inducible gene 6, which in turn reduces the production of pro-

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inflammatory cytokines by suppressing the NF-KB signaling pathway (55). Therefore, it seems that MSC transplantation can be considered as a novel therapeutic approach for the treatment of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and diabetic neuropathies (57). The favorable effects of MSC transplantation could be amplified when combined with resistance training.

In the present study, Vitamin D3 supplementation was also effective in reducing TNF-β. In addition to calcium homeostasis, vitamin D plays a role in insulin synthesis and secretion as well as regulation of inflammatory and immune responses, cell division, and maturation (58). Diabetes is accompanied by a rise in inflammatory factors TNF-α, TNF-β, IL-6, and high-sensitivity CRP (1,48).

Vitamin D can reduce insulin resistance and increase insulin secretion by regulating inflammatory and immune processes (23,59). Some of the non-classical roles of vitamin D suggest the possible involvement of this molecule in the pathogenesis of T2DM (46). Interestingly, vitamin D has been reported to reduce the production of several cytokines, including IL-2, IL-6, IL-12, interferon-γ, TNF-α, and TNF-β. Inflammatory factors are often associated with insulin resistance and beta cell dysfunction, both of which are characteristics of T2DM (20,58). In 2005, Zhu et al. showed that 1,25 di-hydroxy-vitamin D can downregulate several genes related to TNF-α (60). On the other hand, Diaza et al. reported that calcitriol can reduce the production of inflammatory cytokines, producing TNF-α and decreasing TNF-α gene levels (61,62).

Overall, the combination of resistance training, MSC transplantation, and vitamin D3 supplementation can be considered an effective solution for reducing cerebral cortex inflammation. This strategy might also have positive systemic effects on inflammatory and immune status.

## Conclusion

In the present study, 6 weeks of progressive resistance training with appropriate intensity, duration, and recovery between training sessions, along with vitamin D3 supplementation and MSC injection, could significantly reduce TNF-β levels in diabetic rats. This could probably have been beneficial for the treatment or management of neurological complications of diabetes. However, further studies are required to confirm these findings.


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## Ethics approval

All experiments were performed in the Animal Ethics Committee of *Islamic Azad University, Sari Branch* (ethical code: IR.IAU.SARI.REC.1398.123).

## Conflict of interest

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

## Author contributions

All authors contributed to designing the study, analyzing and interpreting data, writing the manuscript and approving the final submission.

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## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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