

# Research Article:

## Immunopotentiator Effect of $\alpha$ -Tocopherol on Cytokine Expression in the Lymphocytes in the Elderly People



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## ABSTRACT

**Background:** Aging is associated with attenuation of immune responses. Studies show that old people are vulnerable to infectious diseases such as influenza.  $\alpha$ -Tocopherol as an immunomodulator affects immune responses. In the present study, the effect of  $\alpha$ -tocopherol, on lymphocyte responses i.e. interferon-gamma (IFN- $\gamma$ ), Tumor Necrosis Factor-alpha (TNF- $\alpha$ ), and nuclear factor- $\kappa$ B (NF- $\kappa$ B) in elderly individuals was evaluated.

**Materials and Methods:** Heparinized blood samples were prepared from 10 elderly individuals (n=10, age >80 years) as the experimental group and 10 young individuals (n= 10, 20-40 years) as the control group. The separated Peripheral Blood Mononuclear Cells (PBMCs) of aged and young individuals were used for treatment with 2  $\mu$ g/mL of  $\alpha$ -tocopherol and 2  $\mu$ g/mL of Purified Protein Derivative (PPD) after 12 and 24 h incubation period. After isolation of total RNA and synthesise of cDNA, the gene expressions of IFN- $\gamma$ , TNF- $\alpha$ , and NF- $\kappa$ B were evaluated by real-time PCR method.  $\beta$ -Actin gene was considered as the internal control gene.

**Results:** Results showed that treatment with  $\alpha$ -tocopherol increased the IFN- $\gamma$  expression in old and young lymphocyte groups. The mRNA level of NF- $\kappa$ B increased in the PPD group after 12 h in both old and young groups (P<0.05). There were no alterations in TNF- $\alpha$  expression in both groups.

**Conclusion:** It seems that  $\alpha$ -tocopherol is effective in the promotion of cytokine responses in old individuals and may be useful as a supplement for improving the immune system of elderly people.

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## Introduction

**$\alpha$**

-Tocopherol is an important component of vitamin E (Vit E) and plays fundamental biological roles in the body [1-3]. This substance acts as an antioxidant and protects the body's cells against damage [3]. The increased intake of Vit E boosts the immune system via activation of the phagocytic function of macrophages and antibody production [4, 5]. Besides, the presence of fatty acid in Vit E can modulate functions of the immune system in intercellular communication, the fluidity of cell membrane, and formation of the secondary passanger molecule. Vit E can regulate the immune system in this way [6]. Various studies indicate that  $\alpha$ -tocopherol has immunomodulatory effects to prevent excessive inflammatory reactions in responses to immune cells [7, 8].

$\alpha$ -Tocopherol is the most important factor in having a strong immune system, as well as healthy skin and eyes. However, not all of the benefits and risks of vitamin E are well known [9]. Another important aspect of  $\alpha$ -tocopherol is the anti-aging characteristic which may be dependent on its antioxidant effects [10]. Aging is among the well-known risk factors for most human diseases. Furthermore, out of 150000 annual deaths in the world, two-thirds are due to age-related reasons [11]. Aging may be caused by the damage to the DNA, some processes that shorten the length of the telomere, the activity of telomerase enzyme, genetic factors, and so on [12]. Various organs such as cardiovascular system, immune system, respiratory system, and so on can be influenced by aging [13]. Actually, immunosenescence is identified by a progressive deterioration of the immune system related to aging. Various components of both innate and adaptive immune systems experience aging-related changes, such as alterations in the number of circulating dendritic cells and lymphocytes [14].

The immune system alterations are accompanied by age, which ultimately leads to immune aging. Therefore, the elderly are at risk of infection [15]. Recent reports show that individuals older than 60 to 65 years are highly susceptible to influenza infection due to immune system failure and aging. On the other hand, these vaccines are not always effective [2]. Some studies indicate that innate immune-related functions, phagocytosis, complement activity, and many immune system responses are affected by aging [16]. In this regard, Soo-Jin Oh et al. reported that low activities in immune-related aging could be observed in immune components [14].

Quantity and quality of humoral immune responses are affected by aging, and accordingly the characters and class of produced antibodies would change. Low humoral immune system responses in aged individuals increase susceptibility to infectious diseases [16]. In other words, many changes in the immune system in elderly people are caused by T cells. The phenotype and function of T cells change by passing of time. T cells count reduces from  $3 \times 10^9$  to  $7 \times 10^8$ . Instead, the amount of memory B cells and T cells increase. Unlike naive T cells, CD4<sup>+</sup> memory lymphocytes survive with long-lived homeostatic cytokines [17]. Also, CD4<sup>+</sup> memory T cells in older people weakly respond to antigens compared to younger people [18]. Another prominent change in aging is the accumulation of CD8<sup>+</sup> and CD28<sup>-</sup> T cells. Besides, the accumulation of these T cells occurs after repeated antigen stimulation with the virus, so this population is probably derived from CD8<sup>+</sup> and CD28<sup>+</sup> T cells [19, 20]. We need an immunomodulatory substance to alleviate inflammation and potentiate lymphocyte responses in elderly individuals. Therefore, the present study aims to evaluate the effect of  $\alpha$ -tocopherol on lymphocyte expression in the elderly and young people.

## Materials and Methods

### Sample collection

A total number of 10 elderly individuals older than 80 years and 10 young individuals with the age range of 20 to 40 years were recruited for this study. Voluntaries who participated in this study were 5 men and 5 women in both aged and young groups. Elderly participants received metformin and losartan in this study and young individuals did not take any medications. The general health status of both groups was stable. We obtained consent from all participants in this study.

### Peripheral Blood Mononuclear Cells (PBMCs) isolation

First, 10 mL of heparinized blood was taken from all participants. We used a Ficoll to separate peripheral blood mononuclear cells (PBMCs) from peripheral blood. For this purpose, heparinized blood samples were transferred to a tube containing an equal volume of the Ficoll and centrifuged at 4000 rpm for 15 minutes. PBMCs were located between the Ficoll and the blood serum and then slowly collected. The obtained PBMCs were mixed with the RPMI-1640 culture medium (BTI, Iran, BD11) to remove the Ficoll and centrifuged at 4000 rpm for 10 minutes. The obtained cells were mixed with RPMI-1640 and centrifuged at 4000 rpm for 10 minutes to remove platelets with PBMCs. The number and amount of PBMCs were determined using Trypan Blue dye.

**Cell culture and treatment with  $\alpha$ -tocopherol and Purified Protein Derivative (PPD)**

In the present study, all cell culture reagents were provided from. The mononuclear cells were extracted from the peripheral blood cultured in 24-well plates and adjusted in  $1 \times 10^6$  cells/well. Then, the cells were treated with  $2 \mu\text{g/mL}$  of  $\alpha$ -tocopherol (Sigma Aldrich, USA, Cat No. T-3251) and Purified Protein Derivative (PPD) (Razi Institute, Iran). After 12 h and 24 h, RNA was extracted and transcribed to cDNA.

**RNA extraction, reverse transcription, and real-time PCR**

Total RNA was extracted using Yekta Tajhiz kit (Cat No. YT9080) according to the manufacturer’s protocol. To remove any contamination of DNA, the extracted RNA was treated with 0.2 U DNase (Yekta Tajhiz, Iran, Cat No. YT9054) at  $37^\circ\text{C}$  for 5 min and followed by 10 min heating at  $60^\circ\text{C}$ . The reverse transcription was performed via AidTM First Strand cDNA Synthesis Kit (Yekta Tajhiz, Iran, Cat No. YT4500) according to the manufacturer’s protocol.

SYBER green method was used for real-time PCR (Yekta Tajhiz, Iran, Cat No. YT4502). All reactions were performed in duplicate. In this study,  $\beta$ -actin was used as a housekeeping gene and interferon-gamma ( $\text{IFN-}\gamma$ ), tumor necrosis factor-alpha ( $\text{TNF-}\alpha$ ), nuclear factor- $\kappa\text{B}$  ( $\text{NF-}\kappa\text{B}$ ), and  $\beta$ -actin gene sequences were obtained from NCBI (National Center for Biotechnology Information). Gene runner software was used for primer design. Finally, the designed primers were blasted in NCBI to check the accuracy and specificity. Primers sequence was brought in the Table 1 and 2.

**Statistical analysis**

Real-time PCR data analysis was performed based on threshold cycle comparison. In this study, the difference between the threshold cycles obtained from the tested samples (drug-treated cells) and control samples (drug-untreated cells) was calculated, and using the  $\Delta\Delta\text{Ct}$  formula. The ratio of the target gene to the reference gene ( $\beta$ -actin) was calculated through  $2^{-\Delta\Delta\text{Ct}}$ . The statistical calculation of this study was performed using SPSS 16 and results were analyzed by one-way ANOVA. The difference in the expression of target genes between control and treatment samples

**Table 1.** Primer sequences of experimental genes

Gene	Primer Sequence
NF- $\kappa\text{B}$	Forward: 5'- AATTGCCCGGCAT -3' Reverse: 5'- TCCCGTAACCGCGTA -3'
TNF- $\alpha$	Forward: 5'- GTCACCTATTGCTGAGCCTCT -3' Reverse: 5'- AGCTTCTTCCCACCCACAAG -3'
IFN- $\gamma$	Forward: 5'- TCAACAAAGCTGATACTCCA -3' Reverse: 5'- TTCTTACAACAAAATCAAATCA -3'
$\beta$ -actin	Forward: 5'- CCTCCTGAGCGCAAGTAC -3' Reverse: 5'- CTGCTTGCTGATCCACATC -3'

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**Table 2.** The PCR reaction was performed by the Rotor-Gene device according to the following protocol

Step	Function	Temperature	Time	Repeat
1	Incubate	95.00	0:10:0	1
2	Incubate	95.00	0:0:20	40
3	Incubate	57.00	0:0:40	40
4	Incubate	72.00	0:0:40	40
4	Scan			1
6	Melting	55°C to 94°C, every 1.0°C		1 second
7	End			

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was calculated with Tukey's HSD post-hoc test statistical method. The information is displayed as Mean±Standard Deviation (SD). P-value<0.05 was considered significant.

**Results**

**IFN-γ gene expression in the young and old groups**

IFN-γ gene expression increased significantly in cells treated with α-tocopherol in young individuals at 24 h compared to 12 hours (P<0.001). After 24 h treatment, a significant increment of IFN-γ cytokine was observed in PBMCs of the young group compared to the all experimental groups at 12 h and 24 h and β-actin reference gene (P<0.004). There was no significant increase in the IFN-γ expression 12-PPD group (P=0.9447). However, in the 12-α-tocopherol group, the expression of IFN-γ gene was significantly increased (2.2 folds) compared to the β-actin reference gene (P=0.0002). Two folds increase were observed in the 12-PPD group as compared to the β-actin reference gene (P=0.0025). But, there was no significant increase in the 24-PPD group compared to the β-actin reference gene (P=0.05). The highest expression of IFN-γ (2.8 folds) was observed in the 24-α-tocopherol group (Figure 1).

There was no significant difference in IFN-γ expression in the 24-α-tocopherol group as compared to the 12-α-tocopherol group (P=0.9197). In the 24-α-tocopherol group, the expression of IFN-γ gene increased significantly (1.75 folds) compared to the β-actin reference gene (P<0.0001). IFN-γ expression did not increase in 24-α-tocopherol and 12-α-tocopherol groups compared

to 24-PPD and 12-PPD groups, respectively (P=0.0895) (P=0.1309). In the 12-α-tocopherol group, the expression of the IFN-γ gene showed 1.63 folds upregulation compared to the β-actin reference gene (P=0.0002). Also, in the 24-PPD group, we observed a remarkable increase in IFN-γ mRNA level versus the β-actin reference gene (P=0.0478). There were no significant differences among the experimental groups as compared to each other (P>0.05).

IFN- γ expression increased 2.8 folds after treatment with α-tocopherol. Gene expression was evaluated using the real-time PCR method. The significance value is considered as less than 0.05.

**NF-κB gene expression in the young and old groups**

There was no significant difference in the mRNA level of NF-κB in the 24-α-tocopherol group compared to the 12-α-tocopherol group (P=0.9263).

In the 12-α-tocopherol group, the expression of the NF-κB gene showed 1.62 folds upregulation compared to the β-actin reference gene (P=0.0093). In 12-PPD and 24-PPD groups, there were 1.86 folds and 1.72 folds increase compared to the β-actin reference gene respectively (P<0.0001) (P=0.0040). There were no significant differences among experimental groups as compared to each other (P>0.05).

There was no significant increase in the expression level of NF-κB in the 24-α-tocopherol group compared to the 12-α-tocopherol group (P=0.4688). In the 24-α-tocopherol group, the expression of NF-κB gene increased (1.7 folds)

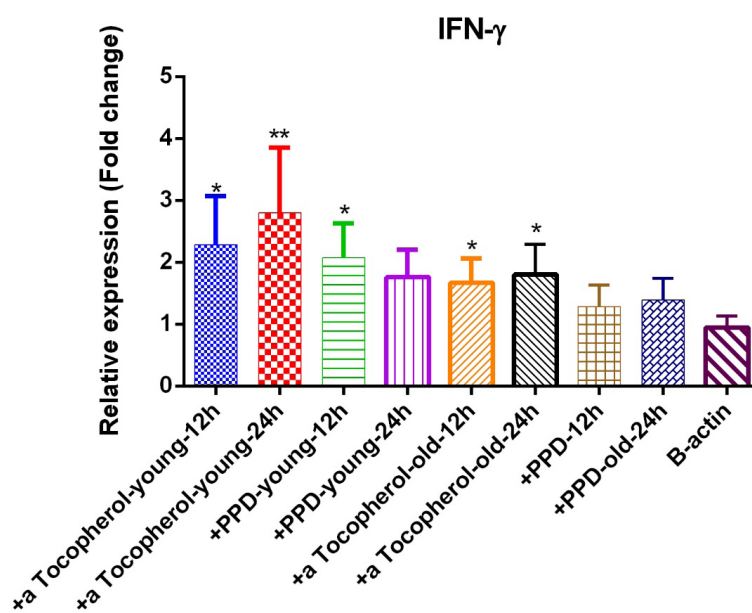
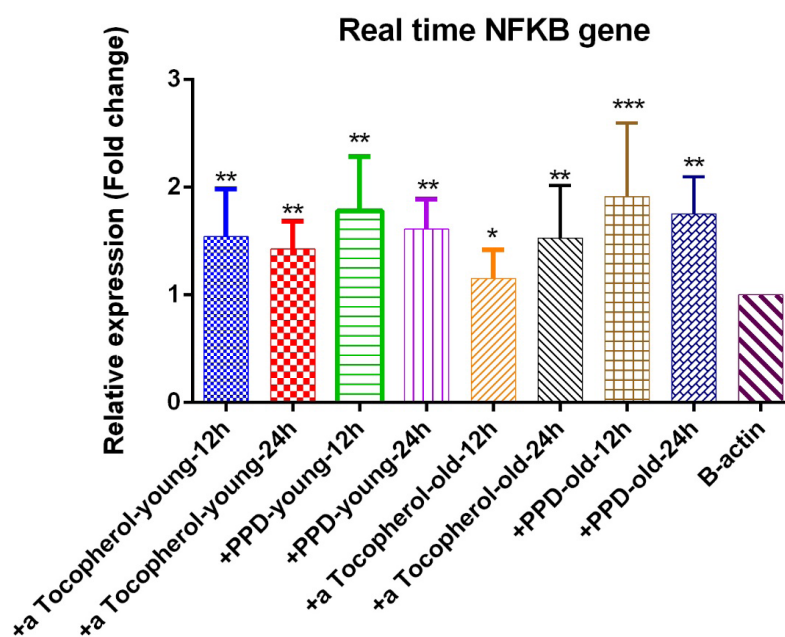


Figure 1. IFN-γ gene expression in the young group



**Figure 2.** NF-κB gene expression in young people

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compared to the β-actin reference gene ( $P=0.0020$ ). The NF-κB gene expression was upregulated 1.9 and 1.88 folds in 12-PPD and 24-PPD groups, respectively compared to the β-actin reference gene ( $P=0.0002$ ) ( $P=0.0003$ ). There were no significant differences among the experimental groups as compared to each other ( $P>0.05$ ).

NF-κB expression increased 1.62 folds after treatment with α-tocopherol. Gene expression was evaluated using the real-time PCR method. The significance value is less than 0.05 (Figure 2).

### TNF-α gene expression in the young group

There was no significant difference in TNF-α expression in the 24-α-tocopherol group as compared to the 12-α-tocopherol group ( $P=0.9993$ ). In the 24-α-tocopherol group, the expression of the TNF-α gene showed a significant increase compared to the 24-PPD group ( $P=0.0274$ ). In the 12-α-tocopherol group, the expression of TNF-α gene did not show a significant increase compared to the β-actin reference gene ( $P=0.5117$ ). There were no significant differences among experimental groups as compared to each other ( $P>0.05$ ).

There is no significant difference in the expression of TNF-α in the 12-α-tocopherol group as compared to the 24-α-tocopherol group ( $P=0.9958$ ). There was no significant difference in TNF-α gene expression versus β-actin reference gene in 12-α-tocopherol and 24-α-tocopherol ( $P=0.8463$ ). The expression of the TNF-α gene in the

12-α-tocopherol group did not show any significant differences compared to 12-PPD and 24-PPD groups ( $P>0.05$ ). There were no significant differences among experimental groups as compared to each other ( $P>0.05$ ) (Figure 3).

TNF-α expression was assessed in the presence of α-tocopherol and PPD for 12 and 24 h treatment time. Gene expression was evaluated using the real-time PCR method. The significance value is considered less than 0.05.

### Discussion

Because the world's population is getting old, it is necessary to improve aged people's immune system and protect them against infections. Aging weakens the immune system which is evident after the age of 60 [15]. The onset of the aging period is from 40 years, after which humoral and cellular immune responses decline gradually. The weakening of the immune system responsiveness in old people makes them vulnerable to viral infections [21]. Vit E is a fat-soluble vitamin that is also known as a powerful anti-oxidant. Antioxidants protect the body's lymphocyte against the damaging effects of free radicals and can reduce the risk of dangerous diseases such as cancer, heart disease, or even Alzheimer [22]. A sufficient amount of Vit E boosts the immune system by increasing the phagocytosis of macrophage and increasing antibody production. Vitamin E is essential for the normal function of the immune system [23]. In this regard, our results showed that IFN-γ gene expression increased in the PBMCs of young individuals after α-tocopherol treatment. In young people, the



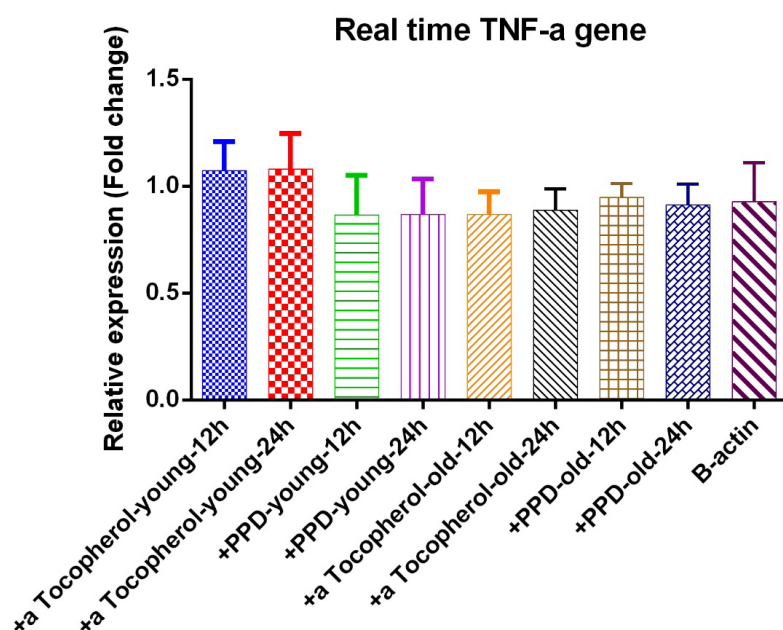


Figure 3. TNF- $\alpha$  gene expression in young people

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increment of IFN- $\gamma$  was observed. But, the important point in these individuals is that expression of IFN- $\gamma$  has significantly increased compared to the control gene after 12 and 24 hours of treatment. These findings show that the elevation of the IFN- $\gamma$  level was affected by  $\alpha$ -tocopherol substance in both young and elderly individuals [24]. Thus,  $\alpha$ -tocopherol potentiates the production of IFN- $\gamma$  by T lymphocyte [25, 26]. Additionally, T cells not only play a role in cellular immunity but are also associated with humoral immune responses by producing high-affinity long-lived antibodies [27, 28]. In this regard, some studies have shown that the consumption of  $\alpha$ -tocopherol in C-57 mice with 22 months of age, which are in the aging stage of life, has been associated with an increase in the expression of IFN- $\gamma$  gene and the findings confirmed our obtained results [29]. Various studies show that the cytokine level of IFN- $\gamma$  decreases in aging [30, 31]. Matthew E. reported that  $\alpha$ -tocopherol reduces inflammatory immune responses. In other words, their results showed that the production of interleukin-12 (IL-12), IFN- $\gamma$ , and NF- $\kappa$ B decreased from macrophages. Therefore, the risk of viral and bacterial infections increases [29]. It is suggested that the cellular immune responses, especially IFN- $\gamma$  cytokine should be potentiated in old individuals. Also, our results showed that NF- $\kappa$ B gene expression increased after 12 and 24 hours of treatment with  $\alpha$ -tocopherol in the PBMCs of young and elderly individuals. This elevation was observed statistically significant after 12 hours of treatment with  $\alpha$ -tocopherol. However, in elderly individuals, NF- $\kappa$ B showed a remarkable increase after 24 hours and there was no significant increment after 12 hours of treatment. NF- $\kappa$ B has various roles in the common part of downstream signaling pathways in inflamma-

tion [32]. It is expressed chronically in the lymphocyte of aged people and is also called inflamm-aging [33]. Chronic low-level inflammation is always present in the body of old people and causes cell death or apoptosis in the face of the antigen [33]. Besides, Suzuki and their colleagues have studied the effect of vitamin E derivatives on the activation of NF- $\kappa$ B caused by tumor necrosis factor. The results of this study demonstrated that consumption of vitamin E could regulate the inflammatory pathway of NF- $\kappa$ B [34].

Considering that IFN- $\gamma$  has increased,  $\alpha$ -tocopherol has been indicated to potentiate the lymphocyte response in the old age group. But it appears that the NF- $\kappa$ B elevation has not stimulated its inflammatory pathway and is followed by other pathways stimulation. For the certainty of this hypothesis, TNF- $\alpha$  was evaluated. Results of TNF- $\alpha$  gene expression in this study showed that the treatment of PBMCs of young individuals with  $\alpha$ -tocopherol and PPD compared to  $\beta$ -actin as a reference gene did not demonstrate any significant elevation.  $\alpha$ -Tocopherol displayed a remarkable effect compared to PPD treatment after 24 hours. The findings show that  $\alpha$ -tocopherol in young PBMCs can increase the expression of TNF- $\alpha$  cytokine. However, there was no increased TNF- $\alpha$  in the PBMCs of aged people treated with  $\alpha$ -tocopherol. As mentioned above, various studies have shown that, in aging, there is a cytokine inflammation in the body—this is called inflamm-aging. Chronic inflammation attenuates the body and reduces immune responses [35]. Therefore, if it is possible to potentiate the immune responses without inflammation in aged people, success is achieved. Results of TNF- $\alpha$  expression demonstrated that

the treatment of the over 80 years old people PBMCs with  $\alpha$ -tocopherol did not affect increasing the expression of TNF- $\alpha$ . It proves that  $\alpha$ -tocopherol does not affect the inflammation process in elderly individuals. Not only it does not promote inflammation but also based on the results of IFN- $\gamma$  and NF- $\kappa$ B in the present project, it can reinforce cellular immunity and other pathways without causing significant inflammation.

Our results support the suitable anti-immunosenescence effects of  $\alpha$ -tocopherol that may improve the lymphocyte function of old individuals. Cytokine results display an immunopotentiator activity of  $\alpha$ -tocopherol that promotes the cellular immune responses in elderly individuals without causing inflammation. It seems that  $\alpha$ -tocopherol can be used as a supplement to potentiate the immune system and can increase the resistance of elderly people against infection.

## Ethical Considerations

### Compliance with ethical guidelines

All ethical principles are considered in this article. The participants were informed about the purpose of the research and its implementation stages. They were also assured about the confidentiality of their information and were free to leave the study whenever they wished, and if desired, the research results would be available to them.

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Bornazist Pajouhan supported this study.

### Authors' contributions

All authors equally contributed in preparing this article.

### Conflicts of interest

All authors declared no conflicts of interest

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