

Research Article



Nicotinic Acetylcholine Receptor Subunit Alpha-7 Mediates PD-L1 and CTLA-4 Expression in HepG2 Cells

Mina Afrashteh Nour^{1,2}, Fatemeh Kheradmand², Yousef Rasmi², Zahra Asadzadeh¹, Mohammad Amin Doustvandi¹, Mahdi Jafarlou¹, Khalil Hajiasgharzadeh^{1,3*}, Behzad Baradaran^{1*}

¹Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

²Department of Clinical Biochemistry, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran

³Connective Tissue Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

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Abstract

Background: The liver is the largest solid organ in the body and has several unique immunological roles. The alpha7 subtype of nicotinic acetylcholine receptor (α 7nAChR) is expressed in the liver and has different immunoregulatory effects. Programmed death ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are two major checkpoints that act as a negative regulator of the immune system. In this study, we examined the effects of activation (with nicotine) and inhibition (with specific siRNA) of α 7nAChR on the PD-L1 and CTLA-4 genes expression.

Methods: Human hepatocellular carcinoma (HepG2) cell line was used to investigate the effects of treatment with low dose (1 μ M) and high dose (10 μ M) of nicotine on the PD-L1 and CTLA-4 expression. In addition to this, the effects of treatment with 100 nM concentration of specific siRNA targeting α 7nAChR (α 7-siRNA) were examined using quantitative real-time PCR.

Results: Compared to untreated cells, the HepG2 cells treated with nicotine exhibited significant dose-dependent decreases in PD-L1 expression. Furthermore, nicotine has a biphasic effect on the CTLA-4 expression and decreased its expression in 10 μ M concentration. The qRT-PCR revealed that α 7-siRNA significantly reduced the mRNA levels of α 7nAChR ($P < 0.01$). Also, compared to control cells, α 7-siRNA treated cells exhibited significant increases in both PD-L1 and CTLA-4 expression.

Conclusion: These experiments determined that nicotine treatment leads to decreases in PD-L1 and CTLA-4 expression. These effects are reversed by treatment with α 7-siRNA, which indicates the involvement of α 7nAChR related mechanisms in these processes.

Introduction

The liver has many different resident immune cells and has several immunological functions, including induction of immune tolerance, strong innate immunity, and poor adaptive immune reactions versus over-reactive autoimmunity.¹ Because of these diverse functions, this organ has been proposed as an immunological organ and performs many essential immune functions.² Immune checkpoint molecules regulate the function of T-lymphocytes and have profound effects on the maintenance of immunological homeostasis.³ To date, nearly 70 membrane proteins have been identified as immune checkpoint molecules.⁴ Among them, programmed death receptor ligand-1 (PD-L1) and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) two most representative immune checkpoint molecules, which negatively regulate

T cell immune function during different phases of T-cell activation.^{5,6} To date, several immune checkpoint inhibitors have been confirmed by the FDA, such as PD-L1 inhibitors (atezolizumab, durvalumab, avelumab), which are used in immunotherapy of diverse autoimmune diseases and cancers.^{7,8}

Nicotinic acetylcholine receptors are made up of five subunits that assemble together to form different pentameric subtypes. In the case of alpha7-subtype of nicotinic acetylcholine receptors (α 7nAChRs) five α 7 subunits assemble around a central ion pore and form α 7 subtype. The stoichiometry of the nAChR subtype in the muscle is a pentamer composed of five subunits (α 1)₂(β)(δ)(ϵ). As some other nicotinic receptors subtype the (α 4)₂(β 2)₃, and (α 3)₃(β 4)₂ have been identified in different organs.⁹ Mounting evidence revealed that α 7nAChRs are

*Corresponding Authors: Khalil Hajiasgharzadeh, Email: hajiasgharzadeh@tbzmed.ac.ir, Behzad Baradaran, Email: baradaranb@tbzmed.ac.ir

expressed in liver cells and play an important role in the modulation of different functions of the liver. The liver is an important organ that is involved in the pathophysiology of systemic inflammation.¹⁰ The $\alpha 7$ nAChR-related mechanisms through the cholinergic anti-inflammatory pathway (CAP) have important intrinsic protective effects in the regulation of immune functions.¹¹ Nicotine as an activator of nicotinic receptors is an addictive substance of tobacco and poses several functions ranging from immunoregulatory to pathological processes.¹² This compound exerts its consequences by binding to nicotinic receptors that are ligand-activated ion channels.^{13,14} Among different subtypes of nicotinic receptors, $\alpha 7$ nAChR appears to be of special significance in several cellular functions. Many different studies indicated that these receptors are functionally expressed in the liver cells, but their exact role in the modulation of liver functions remains to be elucidated.¹⁵

Since the discovery of gene silencing technology by RNA interference (RNAi), extensive studies have been done to explore its therapeutic benefits.¹⁶ Small interfering RNA (siRNA) is a type of RNAi that is produced intracellularly from exogenous synthetic oligonucleotides and can selectively knockdown target gene expression in a sequence-specific manner.¹⁷ Recent advances in designing organ-specific targeted delivery of siRNAs have dramatically enhanced the potency of siRNA-mediated gene silencing that is undergoing clinical development for clinical impact.¹⁸ To date, no study has been undertaken to examine the role of nicotine and its specific receptor in the regulation of liver immune functions. In this study, the benefits, as well as the possible detrimental effects of activation as well as reduced expression of $\alpha 7$ nAChR by its specific siRNA were investigated. It is well known that tumor cells express immunosuppressive checkpoints, including PD-L1 and CTLA-4 enabling their evasion from the antitumor immune responses. Previous studies have shown that nicotine as a carcinogenic compound in cigarette smoke, is involved in the processes of immune system evasion in various types of cancer, including liver cancer. However, the exact intracellular mechanism of the various effects of nicotine is unclear. There are several studies that emphasize the pivotal role of immunosuppressive checkpoints in mediating these effects. Nicotine may affect the processes of immune system evasion of liver cancer cells by altering immunosuppressive checkpoints expression. In this study, we determined the effects of treatment with nicotine on PD-L1 and CTLA-4 expression and aimed to investigate the involvement of $\alpha 7$ nAChR in its effects. Our long-term goal is to enhance our knowledge about the function of this receptor in the liver as well as design novel therapeutic strategies based on RNAi technology for patients suffering from liver diseases. The integrated biology of the function of $\alpha 7$ nAChR represents a new stimulating strategy to the understanding of liver diseases with potential clinical interventions in the future.

Materials and Methods

Main material and reagents

The human hepatocellular carcinoma cell line (HepG2) was obtained from the cell bank at the Pasture Institute (Tehran, Iran) and cultured in RPMI-1640 medium containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin at 37°C in an atmosphere containing 5% CO₂. The RPMI 1640 medium, FBS, trypsin/EDTA, and penicillin/streptomycin mixtures were obtained from Gibco Co. (Gibco, Carlsbad, CA, USA).¹⁹ The rest of the materials including nicotine were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA) unless otherwise specified in the text. Each experiment was repeated three times.

TCGA analysis

The $\alpha 7$ nAChR, PD-L1, and CTLA4 expression data from The Cancer Genome Atlas-Liver Hepatocellular Carcinoma (TCGA-LIHC) project were extracted from the TCGA database (<https://tcga-data.nci.nih.gov/tcga/>). In this TCGA analysis, 374 samples from liver cancer patients and 50 healthy controls were included in this analysis. The expression levels of $\alpha 7$ nAChR, PD-L1, and CTLA4 were analyzed in tumor and healthy groups. The expression levels in tumor samples were compared to those in adjacent normal tissue samples. Also, the correlation analysis between CTLA4 and PDL-1 vs $\alpha 7$ nAChR was performed.

siRNA transfection

To the transfection of siRNA targeting $\alpha 7$ nAChR, HepG2 cells at first seeded in a 6-well plate (the seeding density was 5×10^5 cells per well). The interference fragment of the $\alpha 7$ nAChR gene (sense: 5'- CCAGACAUUCUCCUCUAUA-3) was designed and synthesized by the company Microcynth (AG, Switzerland). Then, the siRNA was transfected following the manufacturer's guidelines using nanoparticles, according to our previous study.²⁰ When the cell density reaches about 75%, the siRNA and nanoparticle mixture were introduced into HepG2 cells. After 6 hours of transfection, the medium was replaced with normal 10% fetal bovine serum, and qRT-PCR was implemented to test transfection efficiency.

RNA isolation, cDNA synthesis, and qRT-PCR

The mRNA expression of $\alpha 7$ nAChR and PD-L1 and CTLA-4 genes were analyzed by qRT-PCR. According to the constructor's protocol, the TRIZOL reagent (GeneAll biotechnology Seoul, Korea) was used to isolate the cells' total RNA. Then, the total RNA concentration was calculated by the Thermo Scientific NanoDrop instrument. The RNA sequence was then reverse-transcribed to cDNA using a Kit obtained from Biofact (Seoul, South Korea). Quantitative reverse transcription PCR was conducted using miScript SYBR Green Kit (Qiagen, Hilden, Germany). 18s was used as a housekeeping gene for

$\alpha 7nAChR$ and PD-L1 and CTLA-4 genes. The expression level of mRNA was evaluated by the $2^{-\Delta\Delta CT}$ method. The primer sequences were obtained from Sinaclon (Tehran, Iran) and reported in Table 1.

Statistical analyses

Processing of all data was via GraphPad Prism 7 software. Three independent experiments were performed in each group, and the mean \pm standard deviation (SD) was used to present the data. Student's t-test for comparison between two groups and one-way analysis of variance (ANOVA) followed by Tukey's test for more than two groups were used to analyze the difference. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

$\alpha 7nAChR$, PD-L1 (CD274), and CTLA4 expression in patients with liver hepatocellular carcinoma (LIHC)

The expression of $\alpha 7nAChR$ in 374 patients with LIHC and 50 healthy controls extracted from the TCGA database (LIHC project) showed a significant increase in the patient's group as compared to healthy controls (Figure 1A). In addition, the results of the TCGA open-source data set indicated that the expression of CD274 was reduced in cancer groups as compared to healthy controls (Figure 1B). The results of TCGA indicated that the expression of CTLA4 was increased in cancer groups as compared to healthy controls (Figure 1C). The correlation

Table 1. The sequence of primers for $\alpha 7nAChR$, PD-L1, CTLA-4, and 18s genes.

Genes		Sequences
$\alpha 7nAChR$	Forward	5' CGCCACATTCCACACTAACG 3'
	Reverse	5' AGACCAGGACCCAACTTCAG 3'
PD-L1	Forward	5' TGCCGACTACAAGCGAATTACTG 3'
	Reverse	5' CTGCTTGCCAGATGACTTCGG 3'
CTLA-4	Forward	5' CATGATGGGAATGAGTTGACC 3'
	Reverse	5' TCAGTCCTGGATAGTGAGGTTTC 3'
18s	Forward	5' ACCCGTTGAACCCATTCTGTA 3'
	Reverse	5' GCCTCACTAAACCATCCAATCGG 3'

analysis between CTLA4 and PDL-1 vs $\alpha 7nAChR$ showed a positive correlation exists between the checkpoints and $\alpha 7nAChR$ (Figure 1D and 1E).

Nicotine downregulates PD-L1 and CTLA-4 expression

The HepG2 cells were examined for PD-L1 and CTLA-4 gene expression using qRT-PCR. We achieved that after 24 h treatment with both low doses (1 μM) and high doses (10 μM) of nicotine the mRNA level of PD-L1 (Figure 2) and CTLA-4 (Figure 3) were downregulated. The high dose (10 μM) of nicotine has reduced the expression of both genes. But, a low dose of nicotine could only reduce the expression of PD-L1 as compared with non-treated cells (Figure 2 and Figure 3).

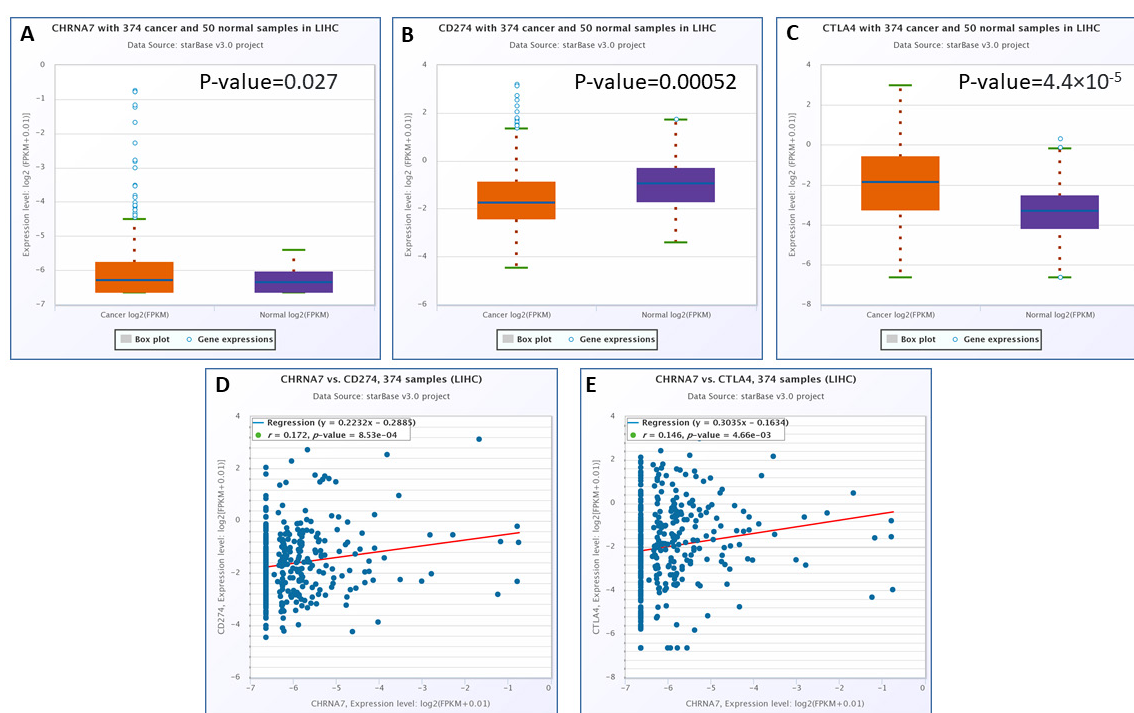


Figure 1. $\alpha 7nAChR$, PD-L1 (CD274), and CTLA4 expression in patients with liver hepatocellular carcinoma (LIHC). (A) The expression of $\alpha 7nAChR$ in 374 patients with LIHC and 50 healthy controls extracted from the TCGA database (LIHC project) showed a significant increase in patients group as compared to healthy controls ($*P < 0.027$). (B) In addition, the results of TCGA open-source data set indicated that the expression of CD274 was reduced in cancer groups as compared to healthy controls ($*P < 0.00052$). (C) The results of TCGA indicated that the expression of CTLA4 was increased in cancer groups as compared to healthy controls ($*P < 4.4 \times 10^{-5}$). (D, E) The correlation analysis between CTLA4 and PDL-1 vs $\alpha 7nAChR$ showed a positive correlation exists between the checkpoints and $\alpha 7nAChR$.

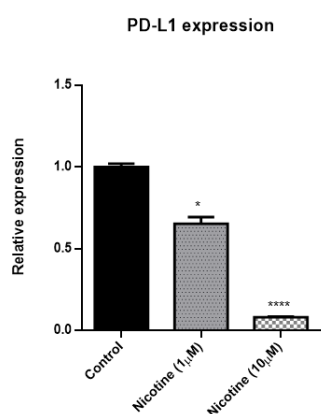


Figure 2. Nicotine leads to the inhibition of the PD-L1 expression of HepG2 cells in dose-dependent patterns. Nicotine treatment with low dose (1 μM) and high dose (10 μM) significantly inhibited the PD-L1 expression. Data are shown as mean ± SEM and all of the experiments were repeated three times. * $P < 0.05$; **** $P < 0.0001$ in comparison with the control group.

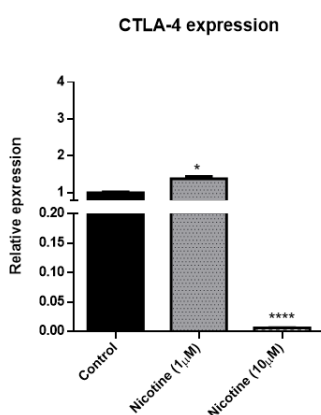


Figure 3. The effects of nicotine on CTLA-4 gene expression. Nicotine treatment with low dose (1 μM) leads to upregulation of CTLA-4 gene expression, but at high dose (10 μM) significantly inhibited the CTLA-4 expression. Data are shown as mean ± SEM and all of the experiments were repeated three times. * $P < 0.05$; **** $P < 0.0001$ in comparison with the control group.

Transfection of $\alpha 7$ nAChR specific siRNA reduced its mRNA expression

The efficiency of siRNA in downregulation of $\alpha 7$ nAChR expression was examined in additional $\alpha 7$ -siRNA treatment groups. The results indicated that $\alpha 7$ -siRNA efficiently suppressed the $\alpha 7$ nAChR expression (Figure 4). Negative control siRNA has no significant effect on $\alpha 7$ nAChR mRNA expression. The results were normalized with the 18s housekeeping gene mRNA level.

Effect of $\alpha 7$ -siRNA on PD-L1 and CTLA-4 expression

We investigated whether $\alpha 7$ -siRNA influences the PD-L1 and CTLA-4 expression in HepG2 cells. For this purpose, additional qRT-PCR experiments were performed to identify the effect of $\alpha 7$ -siRNA on PD-L1 and CTLA-4 expression (Figure 5 and Figure 6). The results showed that $\alpha 7$ -siRNA increased the PD-L1 ($P < 0.01$) and CTLA-4 ($P < 0.0001$) expression level. Similarly in this experiment, were normalized the data with the 18s housekeeping gene mRNA level.

Discussion

The liver is an immunological organ with different exquisite mechanisms of immune regulation to ensure effective immune responses against pathogens.²¹ Among the different checkpoint therapies, CTLA-4 and PD-L1 may be the most significant immune checkpoints for preventing autoactivation.²² The reciprocal interaction between the dysregulated function of liver resident immune cells and undesired immune responses may promote the initiation of several diseases in the liver. Rudolph Virchow presented the concept of a link between chronic inflammation and tumor development.²³ Nowadays, it is well-known that the inflammatory reactions in the liver can be self-limited or persistent depending on the etiology, liver health state, the concentration of toxins or pathogens, and the time of exposure to toxins or infection.^{24,25} Considering the importance of regulating immune activity in the liver, the $\alpha 7$ nAChR may play a crucial role in the regulation of inflammation through the CAP in various pathological conditions of the liver.^{26,27} Although smoking is known to be a harmful behavior, most of its effects are due to the presence of chemical compounds such as NNN and NNK.²⁸ It should be distinguished between the effects of various components of cigarette smoke and

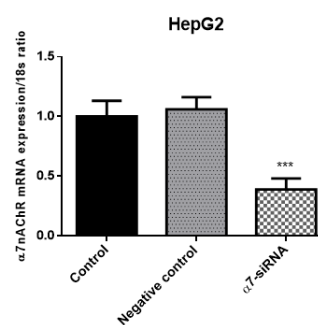


Figure 4. Treatment of the HepG2 cells with the $\alpha 7$ nAChR specific siRNA efficiently reduced the $\alpha 7$ nAChR mRNA expression. 18s was used as an internal control. Data are shown as mean ± SEM and all of the experiments were repeated three times. *** $P < 0.001$ in comparison with the control group.

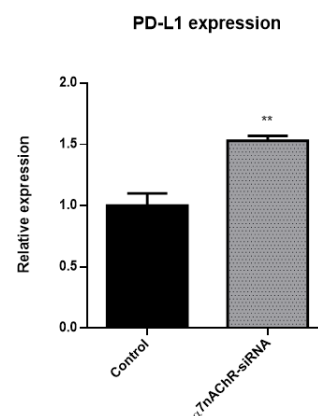


Figure 5. The effects of $\alpha 7$ -siRNA on the PD-L1 gene expression in HepG2 cells were examined by qRT-PCR experiments. The results showed that $\alpha 7$ -siRNA increased the PD-L1 expression level. Data are shown as mean ± SEM and all of the experiments were repeated three times. ** $P < 0.01$ in comparison with the control group.

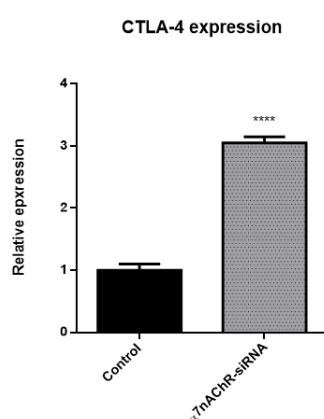


Figure 6. The effects of $\alpha 7$ -siRNA on the CTLA-4 gene expression in HepG2 cells. The results showed that $\alpha 7$ -siRNA increased the CTLA-4 expression level. Data are shown as Mean \pm SEM and all of the experiments were repeated three times. **** $p < 0.0001$ in comparison with the control group.

the effects of pure nicotine. Pure nicotine and other $\alpha 7$ nAChR specific ligands may have therapeutic value in inflammatory diseases, however, the exact effects and therapeutic efficacy of such nicotine-based treatments are inconsistent. Numerous studies have suggested that electrical or pharmacological potentiation of $\alpha 7$ nAChR, using vagus nerve activation or exogenous $\alpha 7$ nAChR agonists (such as nicotine or pharmacological compounds) could represent innovative therapeutic strategies to limit undesired inflammatory cytokines release.²⁹ According to the results of this study, we suggest that activation of $\alpha 7$ nAChR by vagus nerve stimulation (e.g. electrical or pharmacological) or perhaps $\alpha 7$ nAChR agonists (e.g. nicotine, GTS-21, AR-R1779, etc.) may provide innovative interventions for the management of immune system dysfunction or exhaustion in the liver.³⁰ In the present study, the effects of nicotine and its receptor blockade on the PD-L1 and CTLA-4 expression were analyzed and we provide functional evidence that nicotine could reduce the expression of these immune checkpoints through the $\alpha 7$ nAChR in HepG2 cells. Although we used nicotine in this study, recent data have shown that the vagus nerve is not the only source for ACh in the liver, and hepatocytes and other liver resident cells, as well as regulatory T-cells, can synthesize and secrete ACh in this organ.³¹ Therefore, our approach was based on the gene suppression techniques using siRNA technology to inhibit all these possible activations of $\alpha 7$ nAChRs. Also, the role of liver resident immune cells such as Kupffer cells in the diverse functions of $\alpha 7$ nAChR in this organ should be carefully considered when investigating the immunoregulatory effects of nicotine in the liver. Although this study suggests that $\alpha 7$ nAChR is probably a target for the effect of nicotine on PD-L1 and CTLA-4 expression, it is in controversy with our working hypothesis and provides evidence against the direct role of nicotine in the upregulation of immune checkpoints.^{32,33} Also, other studies revealed that the effects of nicotine on immune checkpoints expression were exerted by other types of nicotinic receptors. For instance, Nguyen et al. indicated that $\alpha 9$ nAChR mediates

nicotine-induced PD-L1 expression and regulates skin cancer-related properties.³⁴ In this study, we could not obtain more detailed mechanisms regarding other immune checkpoints molecules expression in HepG2 cells to nicotine exposure, thus further studies are needed to determine the exact intracellular signaling pathways downstream of $\alpha 7$ nAChR in the regulation of immune responses in the liver.

Conclusion

These findings strengthen the hypothesis that modulation of $\alpha 7$ nAChRs may contribute to the regulation of immune functions of the liver. It can be concluded that nicotine could lead to disturbance of the crucial equilibrium in immune checkpoints functions in the liver, which may result in subsequent liver diseases. Our findings suggest caution in the use of cigarettes and other tobacco products that contain nicotine, as they could have a potentially detrimental effect on the regulation of immunological functions of the liver.

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Author contributions

MAN, and KH devised the main conceptual ideas and participated in the design of the work. MAN, ZA, and MAD performed the experiments. MAN and KH wrote the initial draft of the manuscript. FK, YR, MJ, and BB participated in the analysis of the work and reviewed and edited the manuscript.

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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.

Ethical Issues

All experiments and procedures were conducted in compliance with the ethical principles of Tabriz University of Medical Science, Tabriz, Iran and approved by the regional ethical committee for medical research.

Conflict of interest

The authors certify that there is no potential conflict of interest in relation to this article.

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