Iranian Journal of Basic Medical Sciences

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IJBMS

Association of the gene expression variation of tumor necrosis factor-α and expressions changes of dopamine receptor genes in progression of diabetic severe foot ulcers

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ARTICLE INFO

Article type:
Original article

Article history:
Received: May 10, 2017
Accepted: Aug 10, 2017

Keywords:
Diabetic severe foot ulcers
Dopamine receptor
Medical genetics
Regenerative medicine
Tumor necrosis factor-α

ABSTRACT

Objective(s): Regulation of pro-inflammatory factors such as TNF-*α*, which are secreted by the immune cells through induction of their several receptors including dopamine receptors (especially DRD2 and DRD3) is one of the noticeable problems in diabetic severe foot ulcer healing. This study was conducted to evaluate the alteration of TNF-*α* in plasma as well as DRD2 and DRD3 changes in PBMCs of diabetics with severe foot ulcers.

Materials and Methods: Peripheral blood samples were collected from 31 subjects with ulcers, 29 without ulcers, and 25 healthy individuals. Total mRNA was extracted from PBMCs for the study of DRD2, DRD3, and TNF-*α* gene expression variations. Expression patterns of these genes were evaluated by real-time PCR. Consequently, concentration of TNF-*α* was investigated in plasma.

Results: Significant decrease in gene expression and plasma concentration of TNF-*α* in PBMCs was observed in both patient groups at $P<0.05$. These diminutions are correlated to the decrease in the expression of both DRD2 and DRD3 in PBMCs of both patient groups. Also, the same relationship is present between expressions of two new DRD3 transcripts with TNF-*α* downturn.

Conclusion: We concluded that DRD2 and DRD3 expression alteration and presence of new DRD3 transcripts can be effective in reduction of TNF-α expression as a pro-inflammatory factor. Performing complementary studies, may explain that variations in DRD2 and DRD3 are prognostic and effective markers attributed to the development of diabetes severe foot ulcers.

► *Please cite this article as:*
Vaseghi H, Pornour M, Esmaeeli Djavid GhR, Rigi G, Mohammad Ganji Sh, Novin L. Association of the gene expression variation of tumor necrosis factor-α and expressions changes of dopamine receptor genes in progression of diabetic severe foot ulcers. Iran J Basic Med Sci 2017; 20:1213-1219. doi: 10.22038/IJBMS.2017.9475

Introduction

A major health problem in patients with diabetic foot ulcers (approximately 15%) is deficiency in wound healing (1). In the normal process of wound healing, 24 hr after wounding, the expression of proinflammatory cytokines such as IL-1*α*, IL-1*β*, IL-6, IL-12, and especially TNF-*α* is elevated during inflammatory phase of healing and stimulates recruitment of some immune cells including leukocytes, monocytes, neutrophils, and macrophages to remove the bacteria, dead cells, and damaged matrix as well as those involved in bone marrow-derived monocytes/macrophages and leukocytes (respectively T-cells) to the healing site (2-4). TNF-*α* was expressed by both polymorphonuclear leukocytes and macrophages in early phase of wound healing and has expression in the hyper proliferative epithelium at the wound edge (5).

Furthermore, TNF-*α* is involved in stimulation of the expression of growth factors in fibroblasts and keratinocytes as well as up regulation of antimicrobial defenses (3).

After completion of the proliferative phase, TNF-*α* returns to basal levels (2, 6). However, some investigations revealed further levels of TNF-*α* and its impaired effects such as cell migration inhibition of fibroblasts and keratinocytes *in vivo* (2) as well as apoptosis induction on fibroblasts, keratinocytes, and endothelial cells *in vitro* (7, 8). Application of TNF-*α* blocker has been shown to reduce the apoptosis rate, elevated in subject healing (9). In addition to hypoxia and some other conditions contributing to the elevation of the expression of proinflammatory factors, other factors such as TNF-*α* are involved in stimulation of these factors.

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Some studies exhibited the effects of dopamine receptors especially the D2 family (DRD2 and DRD3) on immune cells in line of the expression of proinflammatory and anti-inflammatory factors (10). For instance, selective secretion of TNF-α and IL-10 were triggered in normal human T-cells respectively via DRD3 and DRD2 (10). Dopamine also alters cytokine production especially inflammatory regulator cytokines such as TNF-α, IL-6, CCL2, CXCL8, and IL-10 in monocyte derived macrophages via DRD3 and DRD4 receptors, as well as dopamine transporter (DAT) expressed on their cell membranes in basal and inflammatory conditions (11). Generally, alteration of these receptors has an important role in pathogenesis of various diseases including breast cancer, non-small cell lung cancer, lupus erythromatosus, type II diabetes, *etc* (12-16). In spite of TNF-α importance and functions as a proinflammatory factor in wound healing and the role of dopamine receptors (DRD2 and DRD3) in stimulation of TNF-α, there were no studies on evaluation of TNF-α rate in subjects with ulcers and its association with these receptors in mononuclear cells. Thus, the aim of this study is to evaluate the alteration of TNF-α in serum and dopamine receptors (DRD2 and DRD3) in PBMCs of subjects with ulcers.

Materials and Methods

Sampling

Blood samples (5 ml) were obtained from 31 patients with ulcers (50 to 75 years old), 29 without ulcers as the first control group (50 to 75 years old) and healthy individuals as the second control group (47 to 70 years old) who referred to regeneration group of Academic Center for Education, Culture and Research (ACECR), Tehran, Iran. Patient consent for all samples was obtained according to the Declaration of Helsinki principles. All pathological information of patients was collected from the pathology department of the academic hospitals. The criteria for subjects with ulcers included the patients who had diabetes for more than 10 years and had stage I or II ulcers without necrotic tissue and no infected cartilage. In addition, they did not use antidepressants during the past 6 months and had no addiction to narcotics. It is worth adding that the selection condition used for subject control was similar to patients with ulcers and demographic data of the studied patients were prepared by ACECR. This project was approved by ACECR and a written informed consent was obtained from all participants of this study. Also, this project was approved by the ethics committee of breast cancer institute of ACECR (ethics no. : ir.acecr.ibcrc.rec.1394.38).

Cell isolation

Isolation of cells was performed using PBMCs isolation methods (12) with slight manipulation. 5 ml of peripheral blood was obtained from the cubital vein and collected in cell preparation tubes containing an anticoagulant (heparin). Blood samples were centrifuged at 300 g for 5 min and plasma was preserved for TNF-α assessment. Blood samples were then diluted with an equal volume of Phosphate buffered saline (PBS). PBMCs were isolated from 5 ml of each blood sample by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. Cell density and osmolality were 1.077 ± 0.001 g/ml (20°C) and 290 ±15 moms, respectively. Horizontal swing-out centri- fuge was used at 850 g for 20 min with 1.0 speed regulation for isolation of cells. Buffy coat (lymphocyte layer) was collected and centrifuged at 300 g for 10 min with 2.0 speed regulations and finally, the resulting pellet was washed in PBS (12).

Reverse transcription polymerase chain reaction

Total mRNA was isolated from PBMC using High pure RNA isolation Kit (Roche, Germany), according to the manufacturer's instructions. To synchronize all extracted RNA samples, RNA concentration was read by Nanodrop. The RNA (1 µg) from each sample was used to synthesize the first-strand cDNA by using the cDNA synthesis kit (Fermentase, Germany). Likewise, cDNA synthesis was carried out based on the vendor's protocols. Primers were designed for DRD2, DRD3, TNF-α, and β -actin genes housekeeping gene by using the OLIGO5 software (WWW.oligo.net) based on GenBank sequences. Moreover, BLAST database search against nucleotide reference NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to check their specificity theoretically (Table 1). To evaluate DRD2, DRD3, and TNF-α genes expression in PBMC cells, a routine PCR protocol was conducted for all samples in a final volume of 20 µl including 1 unit of Taq DNA polymerase (Sinagene, Iran). Reaction mixtures contained 2–2.5 mM $MgCl_2$, 0.5 mM each of the dNTPs, 0.8 –1 pM primers, 2.5 µl Taq DNA polymerase

Table 1. Primer sequences used in RT and real-time PCR

Genes	Primers sequences	Accession number	Product length
β-actin-Forward	5'-AGACGCAGGATGGCATGGG-3'	NM_001101.3	161 bp
β-actin -Reverse	5'-GAGACCTTCAACACCCCAGCC-3'		
drd2 -Forward	5'-TGTACAATACGCGCTACAGCTCCA-3'	NM_016574.3	127 bp
drd2 -Reverse	5'-ATGCACTCGTTCTGGTCTGCGTTA-3'		
drd3-Forward	5'-TCTGTGCCATCAGCATAGACAGGT-3'	NM_000796.3	156 bp
drd3-Reverse	5'-TAAAGCCAAACAGAAGAGGGCAGG-3'		
TNF-α –Forward	5'-ATGTTGTAGCAAACCCTCAAGC-3'	NM_000594.3	145 bp
TNF-α-Reverse	5'-AGGACCTGGGAGTAGATGAGG-3'		

(Sinagene, Iran), and 1 µl of cDNA was used as a template in each RT-PCR reaction.

In order to amplify the DRD2, DRD3, TNF-*α*, and β-actin genes, PCR was initiated at 95 °C for 5 min and amplified during 35 cycles at 95 °C for 1 min; 60 °C (DRD2); and 62 °C (DRD3, TNF-*α* and β-actin) for 40 sec and 72 °C for 1 min, which were followed by a final extension step at 72 °C for 10 min. Finally, the PCR products were visualized by gel electrophoresis on a 2% agarose gel. Moreover, positive control amplification was used experimentally to verify the primers.

Real-time PCR

Cyber green fluorgenic nucleotide (Roche kit, Germany) was used for monitoring the cDNA amplification in the process of real-time PCR, which was carried out by using specific primer pairs for DRD2, DRD3, TNF-*α*, and β-actin as the internal control (Corbett, Germany). Reaction was performed in 10 µl of solution consisting of 2 µl FastStart Master solution and 0.3 µM of each primer. A total of 9 µl of the reaction mixture was placed into 0.1 vials, and 1 µl of cDNA was added as a template. Thermal cycling consisted of an initial denaturation step at 95 °C for 10 min followed by an amplification program repeated for 45 cycles. The amplification was performed at 95 °C for 10 sec, 60 °C (DRD2), and 62 °C (DRD3, TNF-*α*, and β-actin) for 10 sec, and 72 °C for 10 sec with a single fluorescence acquisition at the end of the elongation step. The third segment comprised a melting curve program accomplished by default program of the real-time PCR instrument. Melting curve analysis was shown to have only one peak for each reaction and was further confirmed by electrophoresis of PCR products, which displayed only a single band with the expected size.

Sequencing

To confirm the amplified sequences, fragments of DRD2, DRD3, TNF-*α*, and β-actin were sequenced by DNA sequencer ABI 3700 capillary system (Applied Bio System, USA) based on the manufacturer's protocol.

ELISA assay

TNF-*α* assessment kit (REF: BMS223/4CE) (ebioscience, USA) was used to evaluate the plasma concentration of TNF-*α* of each sample. TNF-*α* concentration was evaluated by ELISA assay with the help of ELISA plate reader (ELX800™, USA) based on vendor's protocols. This kit includes human TNF-*α* Standard lyophilized (1000 pg/ml upon recons - titution) to determine the equation of the line. Also, there were two low volume (25–100 pg/ml) and high volume controls (150–400 pg/ml). Finally, according to the manufacturer's protocol, the sensitivity of the kit is suitable for detection of 2.3 pg/ml of TNF-*α*.

Statistical analysis

The number of samples was determined by the Minitab 16.1 software and efficiency of each reaction was precisely evaluated using the Linreg software. Real-time PCR data were analyzed by ΔΔCt, $2^{-\Delta Ct}$, and the Rest method (2005&2009) software. Relative changes in plasma DRD2, DRD3, and TNF-α gene expression in PBMCs were evaluated using the independent t-test. The correlation between the changes in DRD2, DRD3, and TNF-α expression levels in PBMCs, as well as age of patients, were assessed using the SPSS (ver. 18) software and R-Studio 1.0.136. One way ANOVA test was performed to inspect the rate of changes in each gene expression and TNF-α release between the three groups. A *P*-value less than 0.05($P<0.05$) was considered statistically significant.

Results

RT-PCR

In this study, gene expression of dopamine receptors and TNF-α were evaluated in PBMCs of diabetics and healthy individuals. Results signified that both of the dopamine receptors were expressed in PBMCs of diabetic groups, and all of the healthy individuals (Figure 1a). On one hand, almost half of the diabetic group expressed DRD3 fragments with 156 bp length (Figure 1b) but on the other hand, both diabetic groups expressed two new unexpected fragments of DRD3 with lengths of 424 bp and 430 bp, which were amplified by the same primers (Figure 1c).

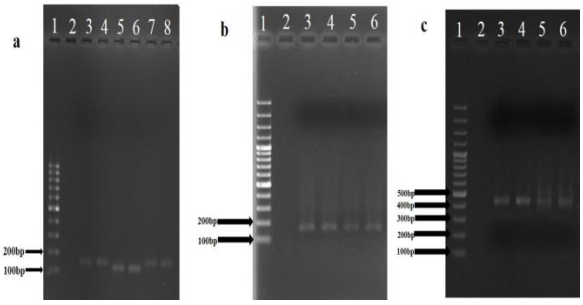

Figure 1. (1a) Expression of DRD2, DRD3, and TNF-α at; (1b) Expression of the expected type of DRD3 fragments (156 bp) in PBMCs of both subject groups; (1c) Expression of an unexpected type of DRD3 fragments (430 bp and 424 bp) in PBMCs of both subject groups.
1a; Lane M: 100 bp size ladder (Fermentase, Germany), Lane 2: control negative, Lanes 3 and 4: DRD3 expression of patient group in PBMCs (156 bp), Lanes 5, 6: DRD2 expression of patient group in PBMCs (127 bp), Lanes 7 and 8: expression of TNF-α in the patient group of PBMCs (146 bp), 1b; Lane M: 100 bp size ladder (Fermentase, Germany), Lane 2: control negative, Lanes 3–6: Expression of the expected DRD3 fragments (156 bp) in both patient groups and healthy individuals of PBMCs, 1c; Lane M: 100 bp size ladder (Fermentase, Germany), Lane 2: control negative, Lanes 3 and 4: expression of the unexpected DRD3 fragments (430 bp) in PBMCs of both patient groups; Lanes 5 and 6: expression of the unexpected DRD3 fragments (424 bp) in PBMCs of both patient groups

Due to designing the primers in CDS and presence of new transcripts with higher dissimilarity with the common sequences in this region, it seems that incidence of the functional changes or expression of new proteins is unavoidable.

Sequencing

Specificity of the amplified fragments (DRD2, DRD3, and TNF-*α*) was confirmed by sequencing. In addition, two new fragments associated with DRD3 in patient groups were sequenced. Sequencing of these fragments revealed two new fragments with a considerable difference between expected DRD3 fragments and two new unexpected fragments. Furthermore, 70 percent identity was detected between the two new fragments. Both of these fragments were submitted to NCBI and recorded with the GenBank accession numbers KX198702 and KX198703 (appendix S1).

Gene expression analysis

In this study, gene expression of the DRD2, DRD3, and TNF-α was evaluated in PBMCs of the patients with and without ulcers as well as healthy individuals. We showed that all of the mentioned genes were expressed in PBMCs of divided groups. Real-time PCR data were analyzed by using the REST software and represented a considerable difference in the genes expression rates between groups (Figure 2). In fact, gene expressions of DRD2 and DRD3 in PBMCs of patients with ulcers displayed a significantly lower expression compared to their counterparts in healthy individuals (Figure 2).

Similar results in the subject group with ulcers demonstrated a reduction in the rate of DRD2 and DRD3 expression in PBMCs of diabetics without ulcers compared to healthy individuals. However, no significant modification in DRD2 and DRD3 expression between patient groups with or without ulcers was identified (Figure 2).

Gene expression analysis of DRD3 was only applied for the patient group who expressed expected and common sequence of this gene. Moreover, transcription of TNF-α had a decreasing rate in PBMCs of the subject group with ulcers compared to subject group without ulcers and healthy individuals; whereas, results revealed an insignificant alteration between subject groups without ulcers and healthy individuals (Figure 2).

The relative expression analysis confirmed the previous analysis, and expression level of each sample was used to determine the decreasing trend of the genes in all three groups. One way ANOVA followed by the *post hoc* test was used to determine the decreasing trends for all of the gene expression modifications. ANOVA analysis disclosed the decreasing trend of DRD2 and DRD3 expression at *P*-value<0.001. However, the *post hoc* test confirmed only a significant reduction in both patient groups compared to healthy individuals (Figure 1S, supplementary data).

Expression of TNF-α revealed the significant drop at *P*-value≤0.022 but, Bonferroni's analysis confirmed a significant change between subjects with ulcers compared to healthy individuals. Furthermore, all sequenced fragments were checked using the BLAST database against the nucleotide reference NCBI database and confirmed the amplicon sequences.

ELISA assay analysis

ELISA assay analysis revealed that plasma concentration of TNF-α had a descending trend in both subject groups compared to healthy individuals (*P*-value<0.05). This result demonstrated that TNF-α concentrations have 26 and 24 percent decreases in patients with and without ulcers compared to healthy individuals, respectively. However, one way ANOVA which was followed by the *post hoc* test (Bonferroni) disclosed that in spite of the descending trend of TNF-α concentrations in healthy individuals and patients

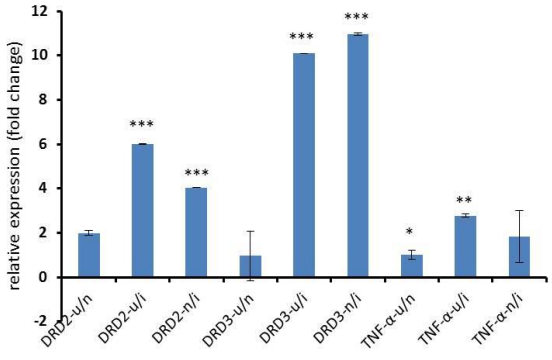

Figure 2. DRD2, DRD3, and TNF-α genes expression reduction in diabetics with ulcers compared to diabetics without ulcers and healthy individuals
U; subject group with ulcers, n; subject group without ulcers, I; healthy individual, ***; significant at *P*-value≤0.001 **; significant at *P*-value≤0.01, *; significant at *P*-value≤0.05

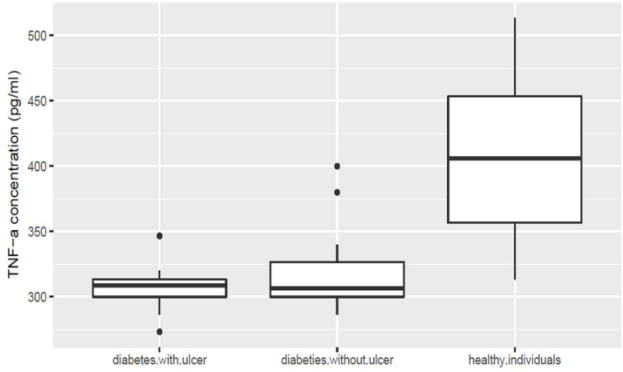

Figure 3. Plasma concentrations of TNF-α in patients with and without ulcers and healthy individuals, X-axis represents the groups and Y-axis shows the mean plasma concentration of TNF-α (mg/ml)

Table 2. Correlation coefficient (r) between dopamine receptors expression (ΔCt) with TNF-α (ΔCt) and age (only in people with diabetes, the expected DRD3 fragment was expressed)

	n	R	*P*-value
TNF-α			
DRD2	58	0.393*	0.038
DRD3	56	0.491**	0.003
Age			
DRD2	36	-0.199 ns	0.330
DRD3	39	0.469 ns	0.177

***; Correlation is significant at *P*-value≤ 0.001, **; Correlation is significant at *P*-value≤ 0.01, *; Correlation is significant at *P*-value≤ 0.05, ns; not significant

without and with ulcers, insignificant changes in the plasma concentrations of TNF-α between subject groups are present (Figure 3).

Statistical analysis

Statistical analysis described significant correlation between decrease in gene expression level of TNF-α and DRD3 (r=0.491, *P*-value≤ 0.003), TNF-α and DRD2 (r=0.393, *P*-value≤ 0.038) and both of dopamine receptors together (r=0.886, *P*-value≤ 0.001) (Table 2).

Furthermore, no considerable variation between the expressions of TNF-α in patients with new DRD3 transcript and common DRD3 transcript in patients with ulcers (*P*-value=0.384) as well as in patients without ulcers was detected (*P*-value=0.458) (Table 2). Further analysis revealed that the TNF-α transcripts decreased significantly in patients with ulcers that expressed two new transcripts of DRD3 compared with healthy individuals at *P*-value< 0.004. Also, such reduction was observed in TNF-α transcripts in patients without ulcers at *P*-value< 0.009. In addition, insignificant correlation between TNF-α and age of both subject groups was found. However, no relationship was found between the gender of patients with the gene expression modifications and TNF-α plasma concentrations in either subject group.

Discussion

Regarding the hypothesis, a significant alteration in plasma concentrations of TNF-α between the subject groups with and without ulcers and healthy individuals is present. Also, a considerable modification was observed in the expression of dopamine receptors (DRD2 and DRD3) in PBMCs of patients with ulcers, and DRD3 had two new different transcripts in the subject group when compared to healthy individuals. Previous studies have explained dopamine receptors particularly on DRD2 and DRD3 potentials in stimulation of pro-inflammatory cytokines such as TNF-α. While most of the studies demonstrated the increased plasma concentration of TNF-α and its adverse effects on patient ulcer healing (17-20), the results of the current study illustrate the decreasing trend of TNF-α in patients with ulcers that have had the disease for more than 10 years and are more than 50 years old. Siqueira *et al.* revealed that dysregulation of TNF-α can be effective in subject wound healing through increased fibroblast proliferation and reduction in their apoptosis rate in mice model of type 2 diabetes(9). The use of the anti-TNF-α antibody in the diabetic mice-impaired wound could improve diabetic wound (20). Also, several investigations revealed that plasma concentration of TNF-α was elevated in insulin resistant people with type 2 diabetes (17, 18). Lixandru *et al.* explained that there was a strong correlation between serum TNF-α and proinsulin concentration in type 2 diabetes (18). TNF-α may stimulate the synthesis of acute-phase proteins that cooperate in the inflammatory process in the vascular wall through induced expression of intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) on endothelial cells as well as being a chemo-attractant for monocytes and other cells contributing in the inflammation process (21). However, some evidence indicated that elevating the dose of TNF-α induces apoptosis, *in vitro* (7-9). To our knowledge, so far there isn't a considerable study demonstrating higher level of proinflammatory cytokines whether in plasma or local in diabetic foot ulcers patients compared to healthy individuals (22). In contrast to the previous studies which showed an increase in the level of TNF-α of patients(7-9), gene expression analysis related to this study revealed a descending trend of TNF-α in mononuclear cell population (the main pro-inflammatory cytokines source) of patients with foot ulcers compared to both patients without ulcers and healthy individuals. Interestingly, the plasma levels of TNF-α in both subject groups were less than those of healthy individuals. The difference between our results in the current study compared to available studies in the database is that most of the previous studies were performed only in different types of subject groups without any individual healthy group as a control (17, 18). Many studies were conducted to show the relationship between insulin resistance, glucose sensitivity, body mass, physiological factors, and otherwise with concentration of TNF-α in patients (17-19, 23). However, Hancock *et al.* revealed that administration of insulin could affect the development of diabetes in NOD mice through disruption of the immune system and it could shift the balance of T-helper cells from Th1 to Th2 pattern including elevation in the rates of IL-4, IL-10, and TGF-β as well as subsiding TNF-α, IL-2, and IFN-**γ** (24). TNF-α has an important role in developing the wound healing process and its concentration for healing in healthy individuals in periodic form has increased pattern for chemotaxis of the different types of cells especially inflammatory cytokines secretor cells (4, 6, 25). So, it seems logical that falling levels of

inflammatory factors such as TNF-α may be the reason for disturbances in chemotaxis and eventually wound healing in diabetes. Dopamine receptors are among the other factors that can induce the secretion of various types of cytokines (10). Modification in the expression profile of dopamine receptors can be effective in pathogenesis of various diseases by inducing changes in physiological and immunological circumstances (12, 14, 15). Specifically, dopamine can induce the secretion of TNF-α, IL-6, and IL-10 in mononuclear cells especially monocytes, macrophages, and T-cells (10, 11). The results of the current study unveiled that, in addition to TNF-α, both DRD2 and DRD3 have expression in PBMCs of patients. However, a significant descendant in their expression rate in patients with and without foot ulcers was identified when compared with healthy individuals. DRD3 has also new sequences differing more in their CDS, which can affect the structural and functional variation compared to its common sequences. Hence, variation in functions is inevitable in patients which express these transcripts. Moreover, significant dipping trend of these receptors has a correlation with dwindling TNF-α expression in mononuclear cells of patients with or without ulcers as well as the descendant rate of TNF-α plasma concentration in these patients. Hereby, it can be explained that expression of TNF-α in subjects is associated with both DRD2 and DRD3 expression alterations as well as the presence of new DRD3 transcript expression. Eventually, these receptors may have a role in wound healing by induction of inflammatory cytokines expression, exclusively TNF-α.

Conclusion

Outcome of the current study, shows the importance of TNF-α in the wound healing process and induction of cell migration, and the potential of DRD2/DRD3 in stimulation of cytokines can explain that expression alteration of DRD2 and DRD3 and the presence of new DRD3 transcripts are prognostic and effective markers attributed to the development of diabetes and their ulcers. Moreover, due to limited blood donation, we cannot perform more studies on the expression of dopamine receptors on the protein level. So, further studies on dopamine receptor changes on the cell surface and variation of local TNF-α and other inflammatory cytokines concentration in patients with ulcers are suggested. However, supplementary investigations using D2 family agonists and antagonists as new therapeutic agents in ulcers healing are recommended.

Acknowledgment

Hajar Vaseghi, Garshasb Rigi and Shahla Mohammad Ganji contributed in PBMCs isolation, RT-PCR, real-time PCR, and ELISA assay. Gholamreza Esmaeeli Djavid and Leila Novin obtained the specimens. Majid Pornour designed the experiments, contributed in performing whole experiments, and analyzed the data. Majid Pornour and Garshasb Rigi wrote the paper.

Conflict of interest

The authors declare no conflicts of interest.

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