Am J Stem Cells 2024;13(4):225-232
www.AJSC.us /ISSN:2160-4150/AJSC0157228

Original Article

Examining the level of inflammatory cytokines TNF-α and IL-8 produced by osteoblasts differentiated from dental pulp stem cells

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Received April 16, 2024; Accepted August 20, 2024; Epub August 25, 2024; Published August 30, 2024

Abstract: Background: The use of dental pulp stem cells (DPSCs) in clinical applications instead of bone marrow stem cells is a very promising method capable of significantly changing the future of medical treatment. If further studies prove that DPSCs and the cells differentiated from them do not stimulate the immune system, these cells can be used more reliably in treatment of autoimmune diseases. Methods: In this research, we examined the isolated DPSCs and differentiated osteoblasts from them in medium without inflammatory stimulants in terms of TLR3 and TLR4 gene expression and inflammatory cytokines, including TNF-α and IL-8 using qRT-PCR, and measured the concentration of inflammatory cytokines IL-8 and TNF-α produced by these two types of cells through ELISA. Results: The obtained results showed that the expression level of inflammatory cytokines IL-8 and TNF-α in differentiated osteoblasts is significantly different as compared with DPSCs. However, no significant difference was observed in TLR-4 expression between two groups. An increase in TNF-α expression level was found to directly correlate with an increase in the expression of IL-8. The concentration of cytokine TNF-α in osteoblasts was significantly higher than that of IL-8 in DPSCs. Conclusion: In comparison to DPSCs, osteoblast cells first lead to inflammatory responses. These responses reduce overtime. However, DPSCs retain their immunomodulatory properties and do not show inflammatory responses.

Keywords: Dental pulp stem cells (DPSCs), osteoblast, differentiated, inflammatory cytokine, Toll-like receptor

Introduction

Mesenchymal stem cells (MSCs) are heterogeneous cells [1] that exist in various body tissues near blood vessels [2]. One of these tissues is dental pulp [3]. DPSCs are capable of regenerating themselves and differentiating into osteoblast cells, chondrocytes, adipocytes, myoblasts and neuroblasts [4]. Unlike mesenchymal cells in other tissues, DPSCs are much easier to access and replaceable [5]. Moreover, they have a higher proliferation rate than other MSCs. These cells have a high expression level of CD73, CD90, and CD105 surface markers and lack expression of CD19, CD14, CD34, CD45, and HLA-DR surface markers [6]. Due to very low immunogenicity and lack of HLA-II expression, rejection of these transplanted cells is much lower than MSCs of other tissues in clinical applications [7].

DPSCs have immunomodulatory properties to modulate and regulate the immune system and express this property through the production and secretion of cytokines, chemokines, and growth factors [8]. For example, DPSCs can secrete IL-10, growth factor TGF-β, prostaglandin E2 (PGE2), indoleamine 2-3 dioxygenase, and nitric oxide [9]. Also, DPSCs directly affect humoral immunity and acquired immunity such that they can inhibit the proliferation of immune cells, B lymphocyte, and T lymphocyte, and direct cell's differentiation toward B reg and T reg cells, respectively [10, 11]. Moreover, proliferation of natural killer cells and neutrophils is inhibited, and macrophages are polarized towards M2 phenotype, which is the inhibitory phenotype [12].

Besides all the research that has been focused on the immunomodulatory properties of DPSCs,

https://doi.org/10.62347/CBMW4366

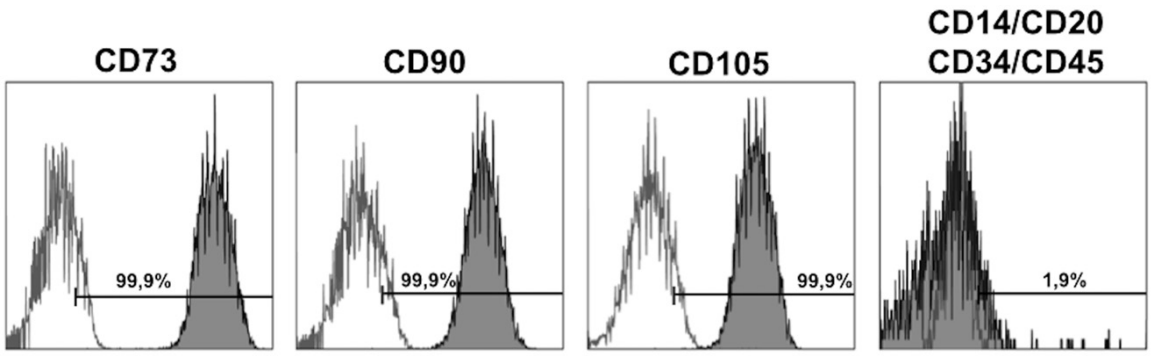

Figure 1. Representative flow cytometry analysis of dental pulp stem cells (DPSCs) after third passage.

scant research has been performed on the immune-stimulatory properties of these cells, as well as osteoblast cells differentiated from them [13, 14]. In this research, we aimed to study the expression and secretion of immune system-stimulating cytokines, such as TNF-α and IL-8 from DPSCs and osteoblast cells differentiated from them. We also explore whether, in terms of Toll-like receptors (TLRs), they are more driven towards the expression of TLR4, immune system stimulation, or the expression of TLR3, immune system suppression. This study aims to investigate whether these cells are potentially and without inflammatory stimulants capable of stimulating the immune system or not.

Finally, if we can conclude that DPSCs and osteoblast cells differentiated from them, do not stimulate the immune system, these cells can potentially be used for autoimmune diseases treatment, stem cell transplants, and differentiation into different cells given their immunomodulatory properties.

Materials and methods

Sample collection and isolation of DPSCs

The studied samples were prepared from six 18-25-year-old people who referred to Shahid Beheshti Dental School for third molar surgery. All patients were healthy based on documented evidence found in their medical files. Each subject was assessed for systemic and oral infection or disease and only disease-free subjects were included in this study. They had no underlying diseases, tooth decay, and dental pulp inflammation. This study has been approved by the ethical committee of the research center of Dental school of Shahid Beheshti University of Medical Sciences. The isolated teeth were disinfected by povidone-iodine solution and kept in PBS containing penicillin and streptomycin (PBS-PS). In order to sterilize teeth, ethyl alcohol was sprayed on them under the hood. To reach the pulp chamber, the surface of each tooth was scraped away using a sterilized high-speed fissure. The healthy coronal pulp tissue was then isolated using a sterile dental excavator. In order to isolate the cells from the pulp tissue, the fragmented tissue should be subjected to 3 ml of type 1 collagenase enzyme for 1 hour at 37°C and high humidity with 5% CO_2. The falcon tube containing cells and enzyme was mixed with gentle shaking every 5 min. For better mixing, the falcon tube was centrifuged at 1200 rpm for 5 min. After centrifuging, the supernatant was discarded and the cells were cultured with 6 ml of complete cell culture medium (D.MEM/F12 + FBS 10% + 100 U/ml Penicillin + 100 mg/ml Streptomycin) in a T25 flask at 37°C, 95% humidity, and 5% CO_2. More detailed analysis was performed once the cells reached the third passage.

Flow cytometry analysis

To ensure whether the isolated cells were DPSCs or not, the flow cytometry test was performed for CD14, CD34, CD45, CD90, CD73 and CD105 antibodies after the third passage (**Figure 1**).

DPSCs differentiation into osteoblasts

In the third passage, DPSCs cells were cultured in two T75 flasks. We selected one of the flasks to be differentiated into osteoblast cells. After the cells were scraped off the bottom of the flask in a checkered plate for 24 h at 37°C with complete cell culture medium, we incubated

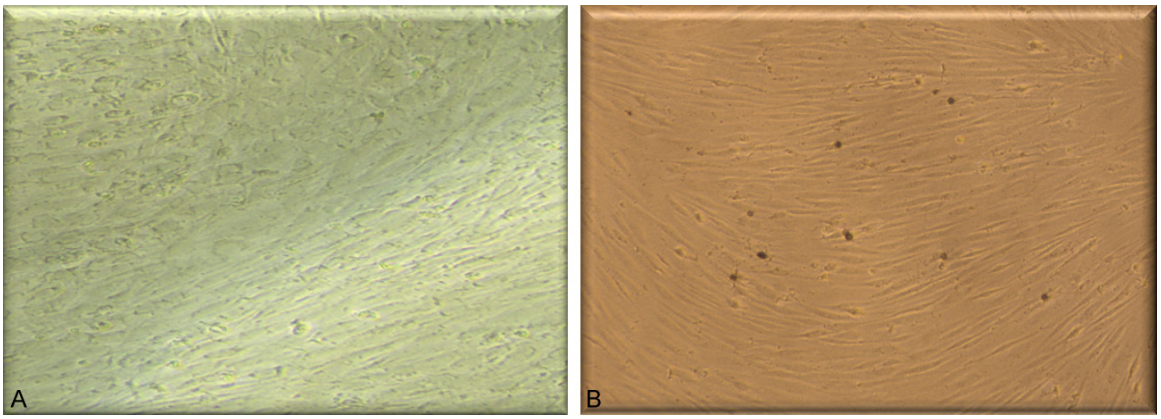

Figure 2. Morphology of dental pulp stem cells (DPSCs) (A). Morphology of osteoblast that be stained by alizarin red (B) (A and B ×100).

the cells. After 24 h, the complete culture medium was replaced with osteogenic culture medium (D.MEM/F12 + FBS 10% + 100 U/ml Penicillin + 100 mg/ml Streptomycin + Glycerol phosphate 10 mM + Dexamethasone 10 mM + Ascorbic acid 2 phosphate 5 mg/ml) and the cells were incubated at 37°C for 21 days. After 21 days, their morphology was studied through an inverted microscope. To ensure the DPSCs differentiation into osteoblast cells, alizarin red staining was performed. For staining, the osteogenic culture medium was first emptied. The cells were then rinsed with PBS and fixed with 1 ml paraformaldehyde 4% and incubated at room temperature for 20 min. They were again rinsed with PBS and stained with the addition of 50 µl of Alizarin red dye for 15 min at room temperature. The cells were then rinsed with PBS to remove the traces of dye. The cells would be studied using an inverted microscope to confirm the DPSCs differentiation into osteoblast cells if red calcium deposits were observed in differentiated cells (**Figure 2**).

Seeding DPSCs and osteoblasts

After DPSCs were differentiated into osteoblast cells, we cultured two groups of cells in a 6-well plate. For DPSCs cells, we considered three wells with times of zero hour before differentiation, 14 days before differentiation, and 21 days before differentiation. We also considered three wells with times of zero hour after differentiation, 14 days after differentiation, and 21 days after differentiation for osteoblast cells. We added 500 µl of complete cell culture medium to three wells containing DPSCs, and 500 µl of osteogenic culture medium to three wells containing osteoblast cells. We then added 2.5 µl and 12 µl cells to wells for DPSCs group and osteoblast cells group, respectively. As osteoblast cells are exposed to osteogenic culture medium, they grow slower and we had better culture more osteoblast cells as compared with the other group.

ELISA

The supernatant was collected at the given times from all 6 wells and stored in labeled microtubes at -20°C for measure of IL-8 and TNF-α through ELISA assay. ELISA kits for TNF-α and IL-8 cytokines were purchased from BioGene CO. (China), and for both cytokines, measurements were performed according to the instructions of the kits. The 96-well plates in these kits were precoated. According to the instructions in the kit, the standard stock solution was diluted to seven different solutions of standards with dilutions of 100, 500, 250, 125, 5, 62, 2, 31, 6, and 15, respectively. Additionally, two wells were considered for positive and negative control. A volume of 100 µl of prepared sample, standards, and positive and negative controls was added in the given plate wells and incubated for 1 h at 37°C. It was then rinsed with a wash buffer and 90 µl color developing reagent was added to the wells and incubated for 30 min at 37°C. The wells were washed again and 50 µl stop solution was added. The absorbance at a wavelength of 450 nm was read by ELISA reader once the color changed from blue to yellow (TECAN, Salzburg, Austria). We exactly repeated the abovemen-

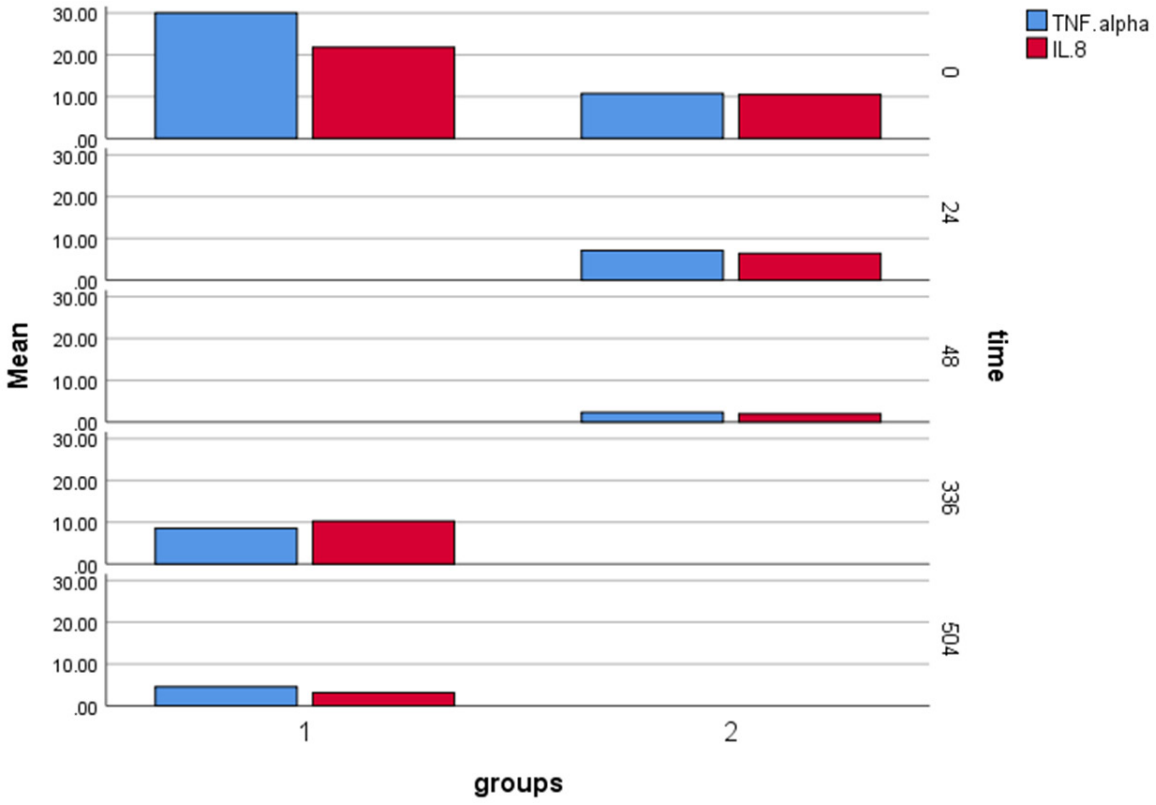

Figure 3. Production of IL-8 (red) and TNF-alpha (blue) by dental pulp stem cells (DPSCs) (1), after 0, 336 hours (14 days) and 504 hours (21 days) before differentiation and by Osteoblasts after 0, 24 and 48 hours after differentiation.

tioned steps for the TNF-α cytokine ELISA test (**Figure 3**).

Real-time PCR

A Real-Time PCR test was used to determine the expression of TNF-α, IL-8, TLR-4 and TLR-3 genes. To this purpose, all cells were, at first, scraped off the bottom of all wells. In order to homogenize the cells, 1 ml of TRizol solution was added to the cells inside the microtube and it was incubated for 5 min at room temperature (Yekta Tajhiz Azma, Tehran, Iran). After that, 200 µl of chloroform was added to the microtube and mixed well until the solution turned milky. It was then centrifuged for 15 min at 1200 rpm and the upper aqueous phase of the microtube containing RNA was separated. After that, 70% ethanol was added to it for washing and allowed to evaporate after centrifugation. In order to measure RNA concentration, the 260/280 ratio was calculated by spectrophotometer. The RT.PCR kit was then used to convert RNA to cDNA (BioFact™, South Korea). According to the kit's instructions, 2 µl of primary RNA was mixed with the first master mix (1 µl Primer + 10.4 µl DDW) and incubated at 75°C for 5 min. Next, 6.5 µl of the second master mix (4 µl Standard buffer + 1 µl dNTP + 1 µl M-MLV + 0.5 Dnase) was added to the microtube and cDNA was fabricated by PCR. For Real-Time PCR test, 1 µl of cDNA was mixed with 19 µl of master mix (Real QPlus 2× Master Mix Green 10 *µl* + 0.5 Forward primer + 0.5 µl Reverse primer + 8 *µl* Nuclease) and PCR machine was set up on Real Time PCR instrument (**Table 1**).

Statistical analysis

Each test was performed in duplicate. The statistical analyses were performed with IBM SPSS Version 25 software. Due to the small sample size, non-parametric Kruskal Wallis test was used in this research. The Games-Howell post-hoc test was used to examine the pairwise differences between groups. A *p*-value less than 0.05 was considered statistically significant.

Table 1. Sequences of primers used for Real-Time PCR

Primer	Sequence (5'-3')
GAPDH	Forward: TTGCCCTCAACGACCACTTT
	Reverse: TGGTCCAGGGGTCTTACTCC
TNF-alpha	Forward: CCTCTCTCTAATCAGCCCTCTG
	Reverse: GAGGACCTGGGAGTAGATGAG
IL-8	Forward: GCTCTGTGTGAAGGTGCAG
	Reverse: CCACTCTCAATCACTCTCAGTTC
TLR-3	Forward: GGACTTTGAGGCGGGTGTT
	Reverse: TGTTGAACTGCATGATGTACCTTGA
TLR-4	Forward: AGAATGCTAAGGTTGCCGCT
	Reverse: CTATCACCGTCTGACCGAGC

Results

Evaluation of cytokines concentrations

The secretion level of two pro-inflammatory cytokines (IL-8 and TNF-α) are evaluated through ELISA test. Obtained data revealed that the secretion levels of IL-8 and TNF-α were significantly increased in osteoblast cells at time zero after differentiation and the cytokine values were decreased after that, after 24 h and 48 h. There was a high concentration of TNF-α in osteoblast cells at time zero after differentiation, and the concentration of TNF-α was decreased and then increased a little again 24 h and 48 h after differentiation, respectively. Also, there was no significant alteration in concentration of pro-inflammatory cytokine IL-8 and TNF-α in DPSCs cells ($P > 0.05$). Using the Wilcoxon Ranked test, the concentration of TNF-α was compared between two cell groups. The concentration of cytokine TNF-α in osteoblast cells was found to be higher than in DPSCs cells ($P < 0.01$). Moreover, the concentration of IL-8 was compared between two cell groups, and no significant difference was found in the concentration of this cytokine between the two cell groups. Moreover, by conducting Spearman's test, no significant correlation was found between the concentration of IL-8 and TNF-α in both groups (**Figure 3**).

Genes expression assessment

The TLR-4, IL-8, TNF-α, and TLR-3 genes expression was normalized by GAPDH gene. Using Kruskal Wallis and Games-Howell post-hoc tests, the expression of TNF-α gene in osteoblast cells was elevated at time zero after differentiation, to be decreased after 24 h, and to be increased again after 48 h ($P < 0.01$). The expression of TNF-α was not significant in DPSCs, while the gene expression of IL-8 in osteoblast cells showed the highest value at time zero after differentiation and then was decreased ($P < 0.05$). The IL-8 expression in DPSCs was not significant. Moreover, the TLR-4 gene expression in both groups was not significant. The difference in gene expression between two groups was then compared at different time intervals. The expression of TNF-α gene in the osteoblast cells group was found to be higher than the expression of TNF-α gene in the DPSCs group ($P < 0.005$). Moreover, the expression of IL-8 gene in the osteoblast cells group was found to be less than this gene expression in the DPSCs group ($P < 0.05$). There was no significant difference in the TLR-4 gene expression between the two cell groups. By conducting Spearman's test, a statistically significant positive correlation was found between the expression of IL-8 and TNF-α in both groups ($P < 0.05$) (**Figure 4**). However, there was no significant correlation between the expression of TNF-α and TLR-4 genes, as well as between the expression of IL-8 and TLR-4 genes in both groups ($P < 0.05$) (**Figure 5**).

Discussion

In this study, it was found that the expression and secretion levels of pro-inflammatory cytokine TNF-α significantly increased in the osteoblast cells group, especially at time zero after differentiation, and after that it is decreased, while this significant increase in the DPSC group was not observed. In line with our research, Viveiros et al. (2022) stated that 3, 5, and 7 days after cultivation, the TNF-α and IFN-γ are not present in the human MSCs medium [15]. Moreover, Hoogduijn et al. (2013) reported that after injecting MSCs to the C57BL/6 mouse model, these cells cause a temporal inflammatory response before inducing immunomodulatory effects, which is specified with an increase in the expression of TNF-α, IL-1β, and MCP-1 [16].

One of the other important points in this research was the significant increase in the expression and concentration of IL-8 cytokine in the osteoblast cells group. The greatest increase in this cytokine was observed at time zero after differentiation, and then, the expres-

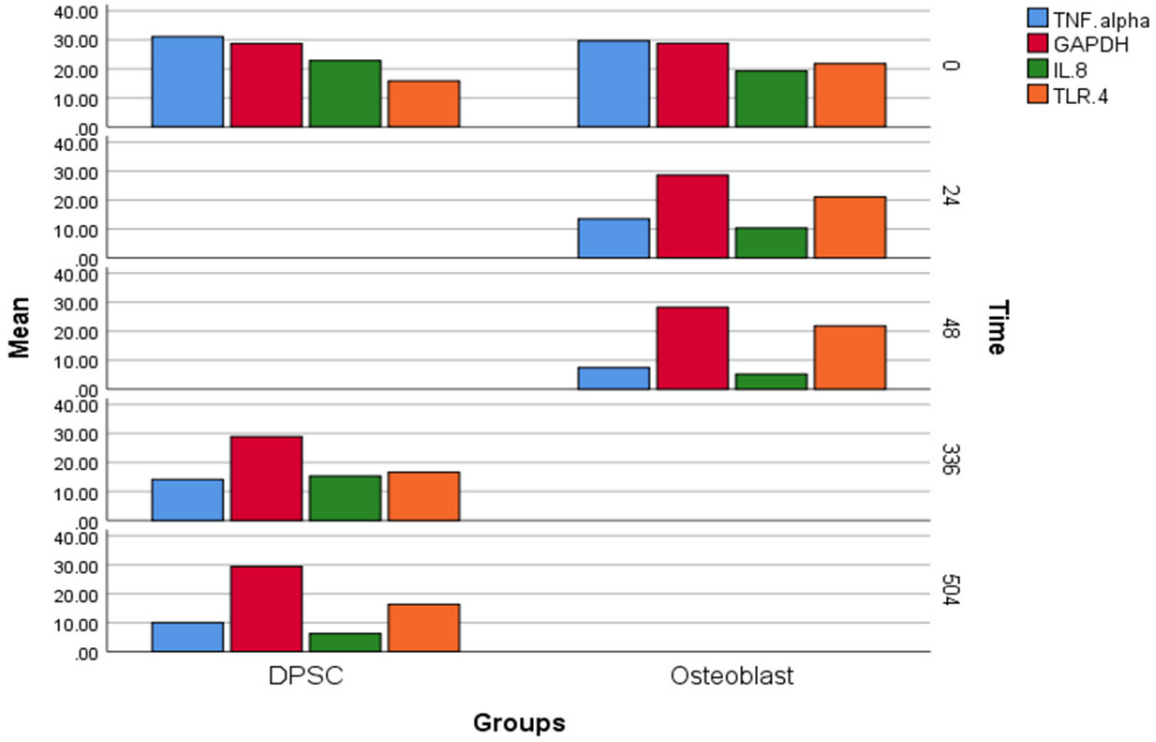

Figure 4. Expression of TNF-alpha and IL-8 in dental pulp stem cells (DPSCs), after 0, 336 hours (14 days) and 504 hours (21 days) before differentiation and in Osteoblasts after 0, 24 and 48 hours after differentiation.

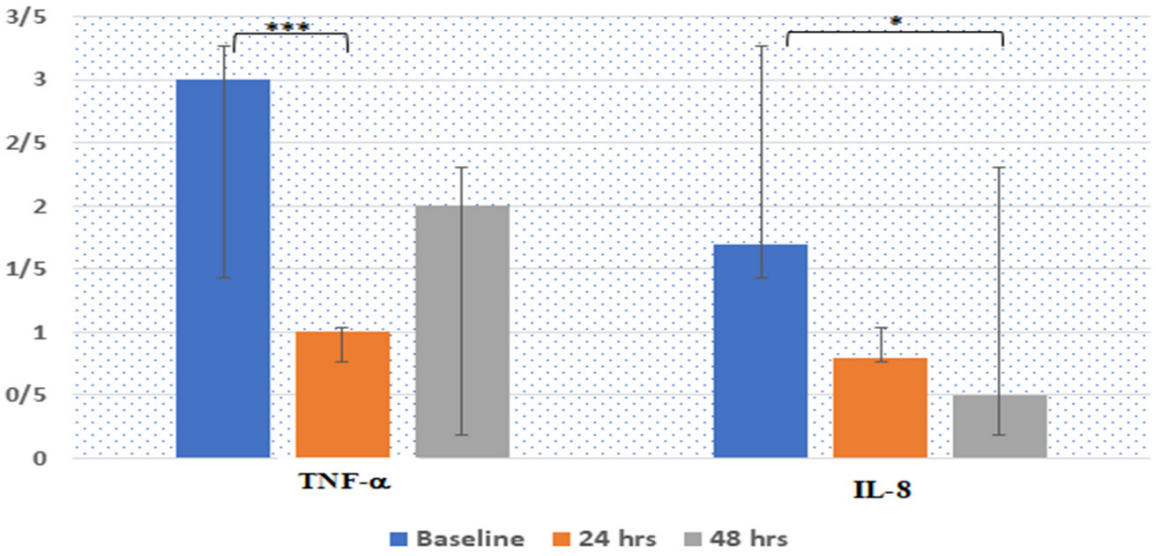

Figure 5. Fold change of TNF-alpha and IL-8 in Osteoblasts after 0, 24 and 48 hours after differentiation.

sion and secretion of this cytokine decreased, while the IL-8 gene expression showed no significant difference in DPSCs at different times. However, the concentration of IL-8 cytokine in the DPSCs group was higher than in the osteoblast cells group. Caseiro et al. (2019), compared stem cells derived from the umbilical cord and DPSCs. They reported the secretion of IL-8 to be significantly higher in stem cells derived from the umbilical cord than in DPSCs [17]. Similarly, He et al. (2013) reported that following treatment with LPS, DPSCs can express IL-8 gene through TLR4, MyD88, NF-κB and MAPK pathways [18]. The results of our study

are slightly different because we did not use any inflammatory stimulant.

In this study, an increase in the expression and secretion of the inflammatory cytokine TNF-α was found to directly and significantly increase the expression and secretion of the IL-8 cytokine. Tsai et al. (2022) reported that TNF-α induces the secretion of IL-6, IL-8, and MCP-1 from differentiated DPSCs, and TNF-α can stimulate inflammatory responses by DPSCs [19]. The results from this study are in accordance with those obtained by our research. Arora et al. (2022) reported that pulp stem cells of decayed deciduous tooth express higher levels of TNF-α and IL-8 as compared with decay free DPSCs [20].

Conclusion

It can be concluded from this research that DPSCs express and secrete some inflammatory cytokines, including TNF-α and IL-8, at the beginning of differentiation into osteoblasts. This significant increase in IL-8 is due to an increase in TNF-α and its direct effect on inflammatory cytokines. However, this does not indicate the immunostimulatory property of DPSCs because the phenotype and behavior of the cells is further affected by its surrounding microenvironment and the concentration of TNF-α has a significant effect on cell behavior. This also holds true for PGE2 and IL-6. The lack of TLR3 receptor expression can be explained by the fact that the TLR-3 signaling pathway in the cell is the TRIF pathway and this pathway ultimately produces cytokines that modulate type I interferons, TLR3 receptor is not expressed with the expression of TNF-α cytokine. On the other hand, as the TLR-4 receptor signaling pathway is both TRIF and MYD88 signaling pathways, TLR-4 is expressed due to the effects of TNF-α and provides signal to the cell through the MYD88 pathway.

Still, further research with longer time intervals, more different types of stem cells, and inflammatory stimulant usage is required for multilateral investigation of signaling pathways that lead to cell differentiation.

Acknowledgements

This article has been extracted the thesis written by Sahar Khastar in school of medicine, Shahid Beheshti University of Medical Sciences.

This work was supported by the school of advanced technologies in medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran (Grant Number: 9208).

Disclosure of conflict of interest

None.

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