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Effects of Lipopolysaccharide from *Porphyromonas gingivalis* and *Escherichia coli* on Gene Expression Levels of Toll-like Receptors and Inflammatory Cytokines in Human Dental Pulp Stem Cells

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ABSTRACT

Background: Periodontal diseases originate from a group of oral inflammatory infections initiated by oral pathogens. Among these pathogens, Gram-negative bacteria such as *p. gingivalis* play a major role in chronic periodontitis. *P. gingivalis* harbours lipopolysaccharide (LPS) which enables it to attach to TLR2.

Objectives: Evaluating the effects of *P. gingivalis* and *E. coli* LPS on the gene expression of TLRs and inflammatory cytokines in human dental pulp stem cells (hDPSCs).

Methods: We evaluated the expression level of TLR2, TLR4, IL-6, IL-10, and 1L-18 in hDPSCs treated with 1µg/mL of *P. gingivalis* lipopolysaccharide and *E. coli* LPS at three different exposure times using Real-time RT-PCR.

Result: The test group treated with *P. gingivalis* LPS showed a high level of TLR4 expression in 24 hours exposure period and the lowest expression in 48 hours of exposure time. In the case of IL-10, the lowest expression was in the 24 hours exposure period. Although in the *E.coli* LPS treated group, IL-10 showed the highest expression in 24 and lowest in 48 hours exposure period. Moreover, IL-18 in *P. gingivalis* LPS treated group showed a significant difference between 6, 24, and 48-time periods of exposure, but not in the *E. coli* LPS treated group.

Conclusion: Both types of LPS stimulate inflammation through TLR4 expression. *P. gingivalis* LPS performs more potentially than *E. coli* in terms of stimulating inflammation at the first 24 hours of exposure. Nevertheless, our study confirmed that increasing *P. gingivalis* and/or the *E.coli* LPS exposure time, despite acting as an inflammatory stimulator, apparently showed anti-inflammatory properties.

Keywords: Dental pulp, Interleukins, Lipopolysaccharides, Stem cells, Toll-like receptors

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INTRODUCTION

The dental pulp is a connective tissue composed of heterogeneous cell populations (1) consisting of numerous different cells and its health or disease significantly affects the dental treatment plans. Dental pulp stem cells (DPSCs) are one of the most important cells among the rest of the cells (2). DPSCs are considered to be mesenchymal stem cells. DPSCs can transform into various cells which produce particular tissues (3). Bone marrow tissue was the first source of isolated MSCs'. Dental pulp tissue is among the multiple sources that have been reported to contain the MSC-like population (4) (isolated by Grontos et al.) such as amniotic fluid, peripheral blood, adipose tissue, bone, cord blood, cartilage, and muscle tissues. Grontos et al. collected the pulp tissue from the human third molar (5) but it can be collected from wisdom (6) primary teeth (7) apical papillae (8). DPSCs have the intensive capacity for differentiation into neuron-like cells, adipocytic, osteocytic, and chondrocyte cells, as well (5, 9). Like the MSCs, DPSCs have CD markers (CD105, CD73, CD90, CD27, CD29, CD146, CD44, CD271, CD166 and STRO-1); while the hematopoietic surface markers (CD14, CD34, and CD45) are absent (10, 11). These cells have the ability of immuno-suppressive and immune-modulating properties in either *in vitro* or *in vivo* conditions. Similar experimental models of inhibitory activity showed that DPSCs are significantly more powerful than bone marrow-derived stem cells. These cells increase the production of anti-inflammatory cytokines like IL-10, while the production of inflammatory cytokines decreases the immune and inflammatory responses such as IL-2, IL-17, and IFNγ (12, 13). With the emergence of stem cells from pulp tissue, experiments have been done to repair the tissue using stem cells and tissue-engineering sciences (5). In dentistry, considerable research has been done on tissue engineering, which combines biology, chemical engineering, and clinical science, by the use of dental stem cells (14). The therapeutic potential of stem cells in periodontics can help rebuild vital structures such as bone, cement, periodontal ligament fibers, and dental pulp (15).

Bacteria are the most common microorganisms involved in endodontic infections (16). *Porphyromonas gingivalis* and *Escherichia coli* are among the essential gram-negative bacteria causing dental pulp disease (17). *Porphyromonas gingivalis* is the most crucial microbial cause of adult periodontitis and is the most critical anaerobic bacterium in causing gum disease (18). It has some potential pathogens such as cysteine proteins (gingipains), lipopolysaccharide (LPS), capsules, and fimbria (19)ml. *Escherichia coli* is one of the most common gram-negative bacilli isolated from clinical cases and is responsible for hospital-acquired infections (20). The most critical way of causing inflammation in gram-negative bacteria is their lipopolysaccharide or endotoxin, which is a part of the cell wall and acts in maintaining its structure and stability (21, 22). The LPS connects to pattern recognition receptors (PRRs), like TLRs and CD14s, and triggers intracellular signals (19)ml to initiate the innate immune response by recognizing the conserved molecular structures in bacteria. Gram-negative bacteria are capable of activating TLR-4 through their lipopolysaccharides, and Gram-positive bacteria are capable of activating TLR-2 via Lipoteichoic acid (23). In oral inflammatory diseases, the production of pro-inflammatory cytokines like TNF and IL-1b and then the induction of absorption alveolar bone and matrix metalloproteinase production can be stimulated by TLR-2 and TLR-4. DPSCs bind to the *Porphyromonas gingivalis and Escherichia coli* LPS by expressing TLR2 and TLR4 and producing different cytokines (24). Cytokines as immunity protein factors are classified into two groups; anti-inflammatory cytokines (IL-4, IL-13, and IL-10) and inflammatory cytokines (IL-2, IL-8, IFN-γ, IL-6, and

TNF-α) while levels of these cytokines can reflect inflammation (25). IL-6, IL-10, and IL-18 are produced by dental pulp stem cells. IL 6 and 18, including inflammatory interleukins and IL-10, are one of the most important anti-inflammatory cytokines which have a vital part in regulating the immune system (12). IL-6 is a multifunctional cytokine with the ability to regulate immune responses, and acute phase responses can play a severe role in the host defense mechanism. IL-18 causes interferon-gamma activity in association with IL-12 and activates innate and acquired immunity and overproduces them by activating macrophages that disrupt the immune system. Also, IL-18 is expressed in a variety of cells including T cells; B cells, liver macrophages, Kupffer cells, microglial cells, dendritic cells, astrocytes, osteoblasts, and keratinocytes (25).

The purpose of this study was to determine the effects of LPS related to *Escherichia coli* (*E. coli*) and *Porphyromonas gingivalis (P. gingivalis)* on TLR2 and TLR4 expression and to determine the correlation between the expression of these two TLRs with the expression of IL-6, IL-10, and IL-18 In the pulp stem cells of human-impacted teeth (See Figure 1).

MATERIALS AND METHODS

Isolation and Cell Culture of DPSCs

At the outset, under accepted indexes that the ethics committee provided (Ethics code: IR.SBMU.MSP.REC.1397.152) and during routine wisdom teeth extraction at the school of dentistry of Shahid Beheshti University of Medical Sciences, isolation of hDPSCs from impacted third molars of three healthy and without no dental caries male human adults was done in the range of 18-26 years . Then after sterilizing the outer part of the teeth with 70% ethanol; a high-speed handpiece of sterile fissure diamond bur was used for reaching the pulp chamber and creating an access cavity. To digest the pulp tissue, the diced dental pulp tissue was incubated in 3 mg/mL of collagenase type1 (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 1 hour. After centrifuging the cells at 12000 rpm for 5 min, resuspended them in complete culture media including Dulbecco's Modified Eagle's Medium: Ham's F12 (DMEM/F12) (Biosera, England), 10% fetal bovine serum (FBS, GIBCO/BRL, Karlsruhe, Germany), 100 g/mL streptomycin and 100 U/mL penicillin (Biosera, England). In the end, the cells were incubated at 37°C in 5% CO2. All experiments were carried out by using the cells in passage 3 to precise the analysis.

Characterization Analysis of DPSCs

Flow Cytometry

For determining mesenchymal stem cell markers, hDPSCs at passage 3 with 70–80 % confluency were analyzed by flow cytometry to assess the expression of stem cell surface markers of hDPSC, so PerCP, PE, and FITC

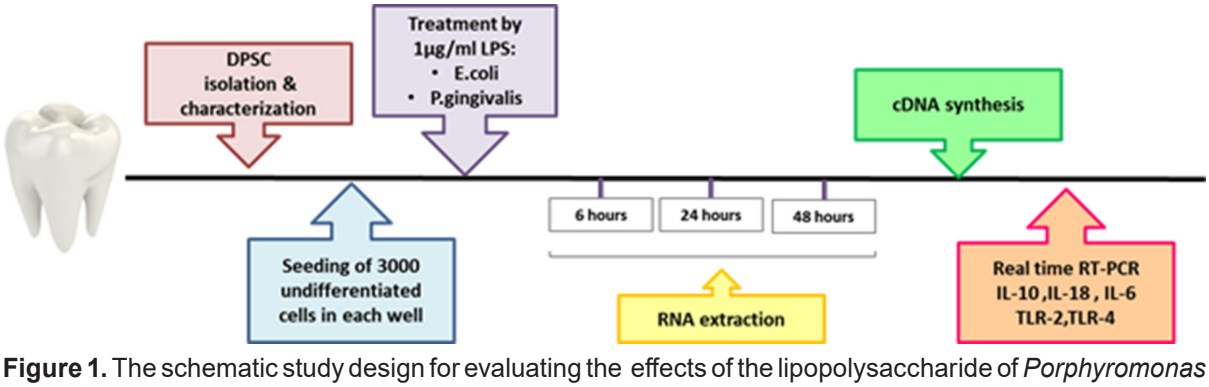

Figure 1. The schematic study design for evaluating the effects of the lipopolysaccharide of *Porphyromonas gingivalis* and *Escherichia coli* on gene expression levels of Toll-like Receptor-2, Toll-like Receptor-4, Interleukin-6, Interleukin-10, Interleukin-18 in human dental pulp stem cells (DPSCs) within 6, 24 and, 48 hours post-treatment.

-conjugated anti-human antibodies which came from Bioscience were used against the appeared markers on cell surface including anti-CD73, anti-CD105, anti-CD34, anti-CD14, anti-CD45, and anti-CD90. To assure the accuracy of the results, three individual tests were performed.

Adipogenic and Osteogenic Differentiation Potential

To define the potential of hDPSCs to differentiate into adipogenic and osteogenic lineages, 3×10^3 cells/well of hDPSC at passage 3 were cultured with DMEM/F12 medium supplemented with 10% FBS (GIBCO/BRL, Karlsruhe, Germany), 100 g/mL streptomycin and 100 U/mL penicillin (Biosera, England) in 24-well plate (SPL). After reaching 70-80% confluency, the previous medium was replaced with the adipogenic medium that contains the compounds of culture media as said before, plus 0.5 mM 3-isobutyl- methylxanthine, 100 mM indomethacin (100 mM), 5 mM insulin, and 250 mM dexamethasone (All from Sigma-Aldrich, Germany). After incubation for 21 days at 37°C and 5% CO2, to examine intracellular oil droplets and assess the differentiation of the cells, oil red-O staining was used. For osteogenic differentiation, 3×10^3cell/well of cultured confluent DPSCs (P3) were incubated with the osteogenic induction medium which consists of 10 mM glycerol phosphate, 10 mM dexamethasone, and ascorbic acid-2 phosphate 5 g/mL (all of them from Sigma-Aldrich, Germany). Also, every 3 to 4 days, the osteogenic medium was exchanged. After the completion of osteogenic differentiation of DPSCs, the cells were stained by using Alizarin Red S to evaluate mineralization (26).

Treatment by LPS

Undifferentiated DPSCs were treated with a concentration of 1µg/mL of 2 different types of LPS; 1) *E. coli* LPS (Sigma. California, USA), 2), *P. gingivalis* LPS (InvivoGen, France) in 3 different times (6, 24, and 48 h). Also, we have undifferentiated and untreated DPSCs for this three times as the control groups.

mRNA Extraction and cDNA Synthesis

To investigate the influence of LPS with the fleeting of time, after stimulation by the *E. coli* and the *P. gingivalis* LPS, 600 µl Trizol reagent (Sinaclon, Iran) was added to extract total RNA after 6 h, 24 h, and 48 h. The samples of RNA were centrifuged (12000 rpm, 15 min, 4°C) after adding 200 µl chloroform. Then, an equal volume of Isopropanol was added to the aqueous transferred phase into an RNase-free 1.5mL tube. In the end, the washed RNA pellet with 70% ethanol was centrifuged (7500 rpm, 8 min, 4°C) and dissolved in 15µl of sterile distilled water. The RNA samples were measured at a 260/280 nm ratio using a spectrophotometer to quantify. According to the manufacturer's instructions, complementary cDNA was synthesized from RNA using a cDNA synthesis kit (Yekta Tajhiz Azma, Iran) in a volume of 20µl reaction mixture. They were stored at -20˚c.

Quantitative Real-time RT-PCR

To evaluate the expression of TLR2, TLR4, IL-6, IL-10, and IL-18 genes in undifferentiated DPSCs, real-time polymerase chain reaction (real-time RT-PCR) was used. Real-time RT-PCR was performed using RealQ Plus 2x Master Mix (SYBR Green) Without ROX (Ampliqon, Denmark). The assay was performed on the Roter Gene 6000 Real-Time PCR System (Qiagen-Germany). Table 1 shows primer sequences for human TLR2, TLR4, IL-6, IL-10, IL-18, and GAPDH genes synthesized by metabion International AG.

All the cycle threshold (Ct) values were obtained in the exponential phase and were normalized by subtraction of the Ct value. We used the method to determine the fold change in the target gene samples.

Data Analysis

The tests were accomplished separately three-fold in duplicate, and all statistics were

Table 1. Primers used for real-time RT-PCR

Primer	Sequence (5′-3′)
hGAPDH	Forward: CCTGCACCACCAACTGCTTA
	Reverse: GGCCATCCACAGTCTTCTGG
TLR2	Forward: GTGACTGCTCGGAGTTCTC
	Reverse: CTTCCTGCCTTCACTTGGT
TLR4	Forward: TGGAAGTTGAACGAATGGAATGT
	Reverse: ACCAGAACTGCTACAACAGATAC
IL-6	Forward: GCACTGGCAGAAAACAACC
	Reverse: GCAAGTCTCCTCATTGAATCC
1L-10	Forward: CAATAAGGTTTCTCAAGGGGCT
	Reverse: AGAACCAAGACCCAGACATCAA
IL-18	Forward: TGATTCTGACTGTAGAGATAATGC
	Reverse: CCTTGATGTTATCAGGAGGATTC

analyzed by IBM SPSS Statistics Version 21.0 (Chicago, IL, USA) and Prism 8.4.3 (GraphPad Software, San Diego, CA). Games-Howell test, Kruskal-Wallis test, and spearman were used to evaluate the means, which counted as statistically significant when p values showed less than 0.05 ($P<0.05$). These values also were represented as the mean±its standard error deviation (SED).

RESULTS

Flow Cytometric Analysis of hDPSCs Surface Markers

The Analysis of surface markers of all 6 expressed on hDPSC (based on mean±SD values of percentage of cells) represented a high expression of tested markers as listed: CD73 (99.03±0.35), CD105 (84.5±7.02),

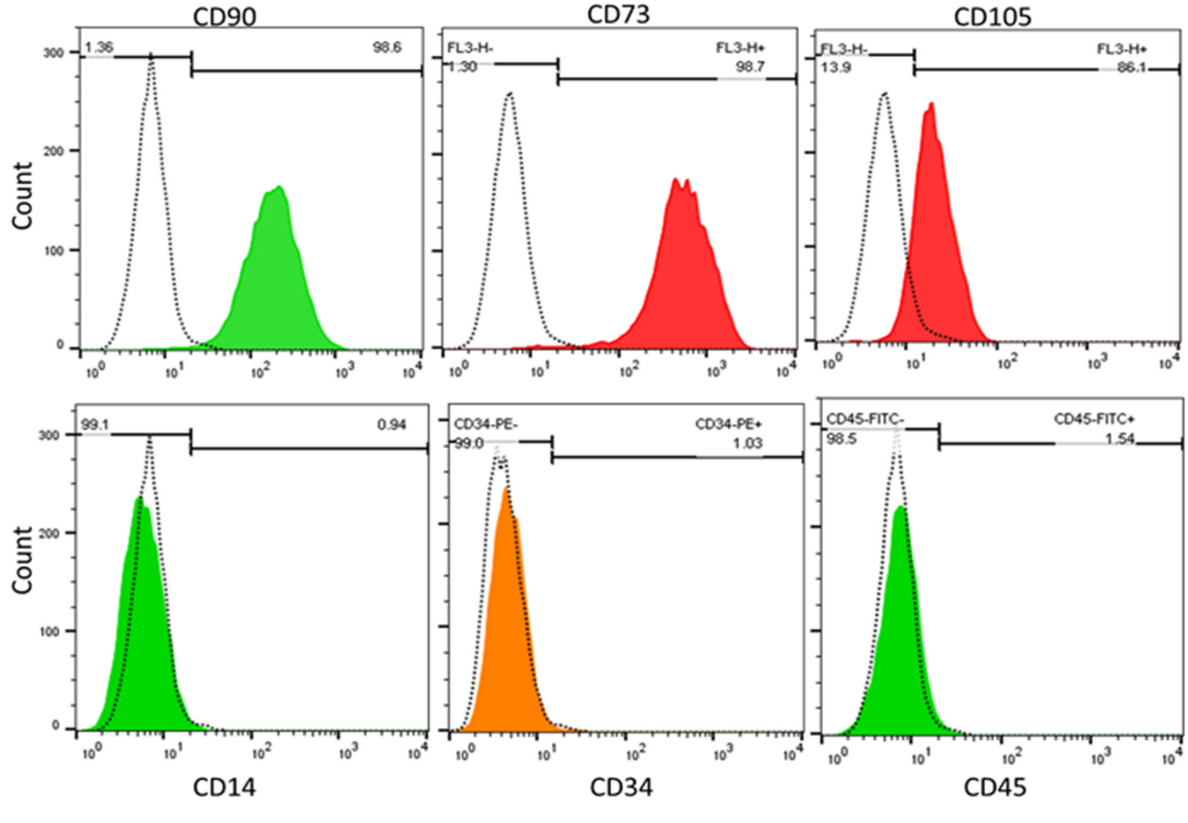

Figure 2. The flow cytometry diagram of human dental pulp stem cells (hDPSCs) surface marker profile with the high expression level of mesenchymal stem cell markers ($CD73^+$, $CD105^+$, and $CD90^+$), and low expression levels of hematopoietic lineage markers ($CD45^-$, $CD34^-$, and $CD14^-$).

and CD90 (98.8±0.81) mesenchymal stem cell markers, while there were lower expression levels of hematopoietic lineage markers including CD45 (1.21±0.66), CD34 (0.94±0.18) and CD14 (1.13±0.78) (Figure 2). Thus, it could be concluded that the DPSCs showed the mesenchymal stem cells immunophenotyping.

Osteoblasts and Adipocytes Differentiation

Three weeks after being cultured in an adipogenic medium, oil droplets were formed in the differentiated hDPSC into adipocytes, and they were positive for oil accumulations (Figure 3B). Moreover, after 21 days, Alizarin Red S staining was used for affirming the existence of hDPSC differentiation osteogenic cells and producing extracellular calcified nodules (Figure 3C). In both tests, no calcium nodule was observed in the control groups.

Gene Expression

The expression of TLR2, TLR4, IL-6, IL-10 and IL-18 genes was evaluated through real-time PCR. For this purpose, the expression of these genes was normalized with the expression of the GAPDH housekeeping gene. After 6 h, the same concentration of both types of LPS had no significant result in the expression of TLR2 and IL-6 genes (P>0.05). While TLR4 expression after 6 hours showed significant differences among all three study groups, yet the lowest expression of TLR4 belongs to the *E. coli* LPS and the highest expression was in the *P. gingivalis* LPS stimulation. Also, IL-10 and IL-18 expression in all the study groups at 6 h intervals presented significant differences. In the *P. gingivalis* group, the expression level of IL-18 gene was comparatively minor than in the *E. coli*, while the expression level of IL-10 gene was suppressed in *E. coli* group and was the highest in the *P. gingivalis* group (Figure 4).

After 24 h, no difference in the expression of TLR2 and IL-10 was observed (P>0.05). During this period, the expression of TLR4, IL-6, and IL-18 in the *P. gingivalis* group was greater than in the control group. However, in

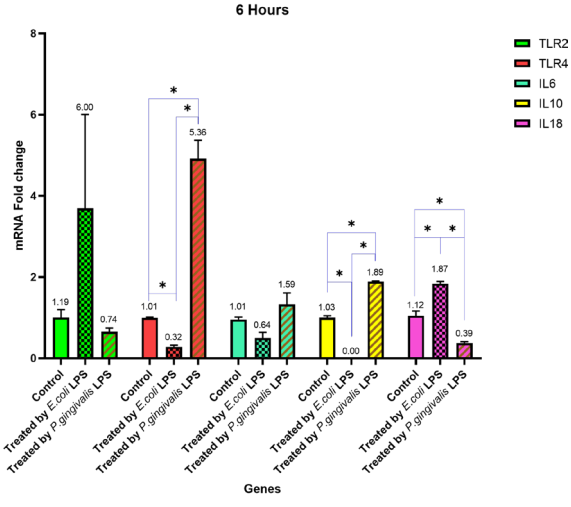

Figure 4. Comparison of mean expression levels of TLR-2, TLR-4, IL-6, IL-10, and IL-18 genes hDPSCs in E. coli, P. gingivalis, and the control groups at 6 h.

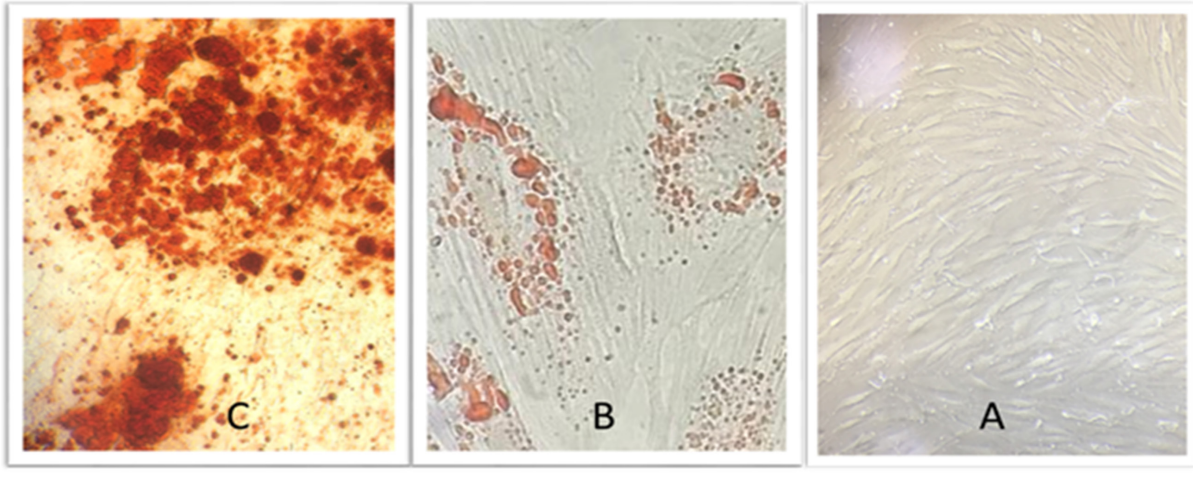

Figure 3. Characterization of hDPSC: (A) morphology of hDPSCs, (B) adipogenic differentiated hDPSCs, oil red-O staining, (C) osteogenic differentiated hDPSCs, Alizarin-red staining.

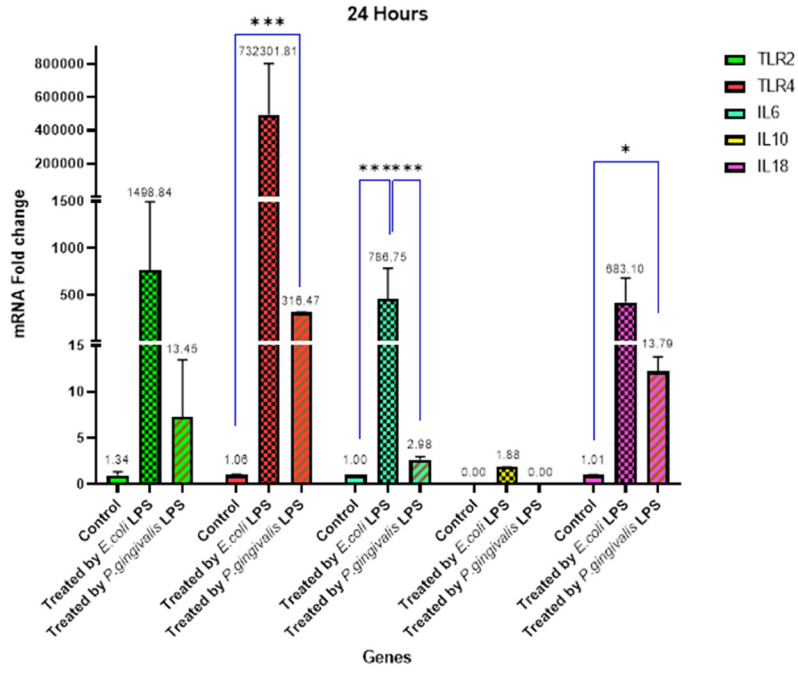

Figure 5. Comparison of mean expression levels of TLR-2, TLR-4, IL-6, IL-10, and IL-18 genes in hDPSCs in E. coli, P. gingivalis, and the control groups at 24 h.

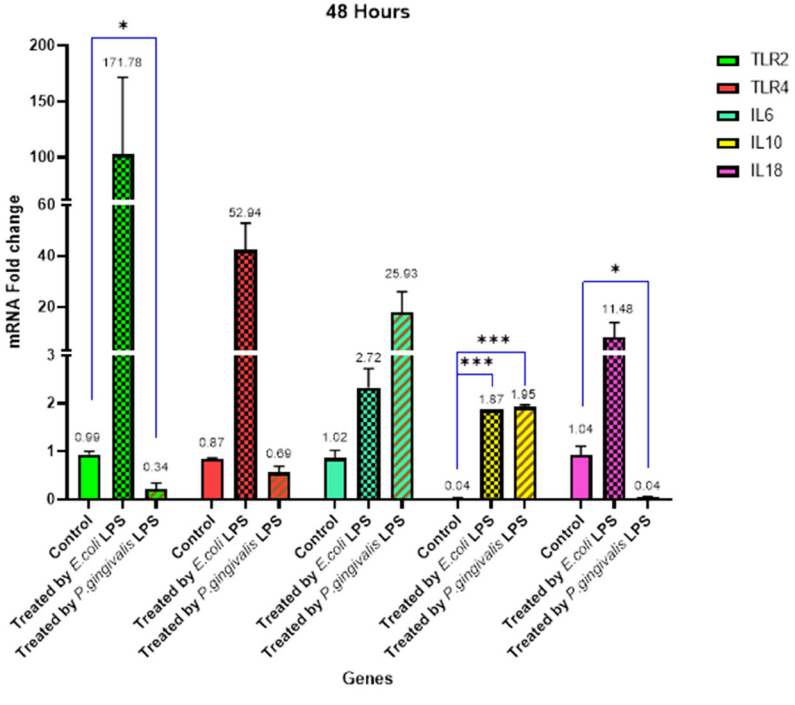

Figure 6. Comparison of mean expression levels of TLR-2, TLR-4, IL-6, IL-10, and IL-18 genes in hDPSCs in E. coli, P. gingivalis, and the control groups at 48 h.

the *P. gingivalis* group, the expression level of all cytokines was lower than *E. coli* group which also shows a significant expression level of TLR4 (Figure 5).

At 48 h, we found only statistically significant differences in the expression of TLR2 and IL-18 between the control and *P. gingivalis* groups, so their expression was remarkably lower in the *P. gingivalis* group. Also, the expression of TLR4 and IL-6 showed no significant difference at this time (P>0.1). The highest expression of IL-

10 belonged to the *P. gingivalis* group and then the *E. coli*, the differences of which were statistically significant in the control group (Figure 6).

Also, we found that when we compare the effects of each LPS on the gene expression level at different times, the *P. gingivalis* effect on induction of TLR4 and IL-18 gene expression between different periods was statistically significant, so the highest-level expression at 24 h and the lowest level at 48 h. Regarding IL-10 gene expression, there was a significant difference between the *P. gingivalis* between 6 and 24 h, as well as between 24 and 48 h, so its minimum expression was related to 24 h. Also, concerning the LPS effect of the *E. coli* on IL-10 gene expression, a statistically significant difference between all the studied times was observed, with the highest expression being at 24 h and the lowest at 6 h. However, the expression level of TLR-2 and IL-6 gene in DPSCs in both groups; the *E. coli* and the *P. gingivalis* in 6, 12, and 24 hours shows no significant difference (P>0.05) (Figure 7A, 7B).

The correlation between TLR-2, IL-6, TLR-4, IL-10, and IL-18 genes in the *E. coli* and the *P. gingivalis* groups was evaluated by the Spearman test. There was a significant relationship between TLR-4 and IL-18 genes as well as between TLR-4 and IL-10 genes in the *P. gingivalis* and the *E. coli* groups.

DISCUSSION

The diagnosis of chronic periodontitis caused by accumulating lots of dental plaque, in its early stages is important to prevent severe

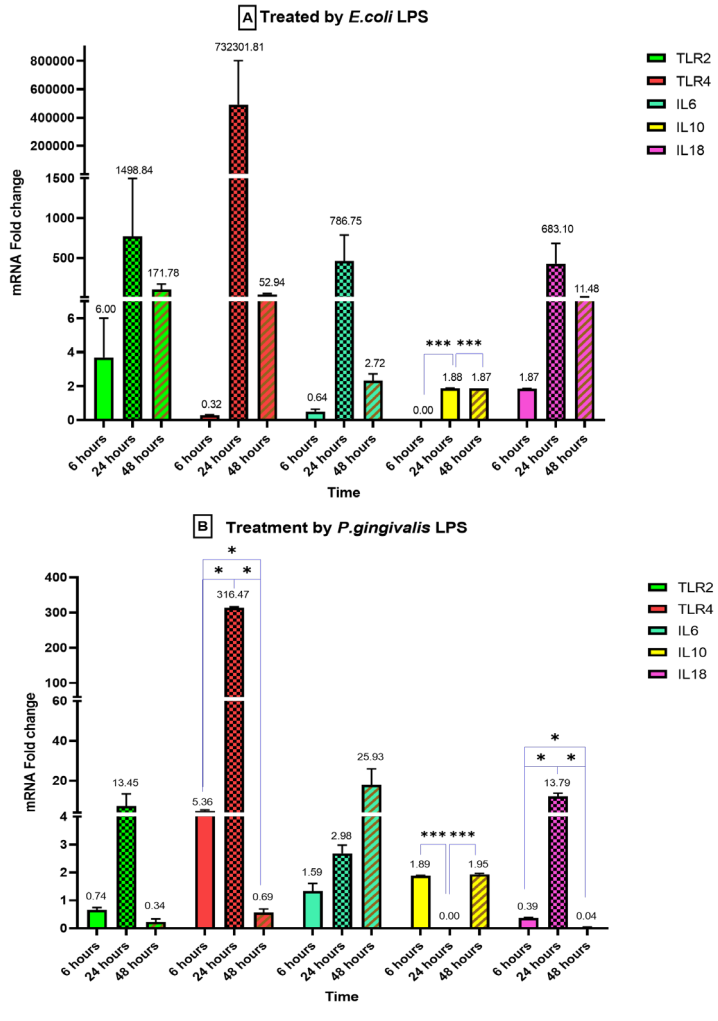

Figure 7. Comparison of mean expression levels of TLR2, TLR-4, IL-6, IL-10, and IL-18 genes in hDPSCs in E. coli and P. gingivalis groups between time points 6, 24, and 48 h.

damage to the protective and dental structures of the teeth (27). One of the major agents in periodontitis is *Porphyromonas gingivalis*, gram-negative oral anaerobic bacteria which can cause tooth loss by exploding the dorsal tissues (28), induce bone resorption by the production of RANKL-induced osteoclasts and activation of Th-17 (29). That infection may affect the development of rheumatoid arthritis (30). Also, its Lipopolysaccharide is the major factor in the development of periodontitis (31, 32) and triggers the destruction of periodontal tissue by the production of inflammatory cytokines like IL-8, IL-1*β*, IL-6, and TNF-α (33). These inflammatory responses were induced via interaction with TLRs and the lipid A component of LPS binding to MD2/ TLR4. However, the *P. gingivalis* LPS was diagnosed by TLR2-CD14, too (34). Bohan Yu et al. discovered LPS-induced TLR4 signaling in hPDLSCs, which caused the osteogenic differentiation of hPDLSCs and the production of inflammatory cytokines (TNF-α and IL-1β) (35). Tamara Kukolj et al. also found that concentrations of 100 to 1000 ng/mL of *E. coli* LPS favored conditions for the differentiation of periodontal ligament stem cells to adipogenic lineage (36). Although in this study, we have a lower concentration of *P. gingivalis* LPS, the results are quite similar in higher expression of TLR4, and IL-18 in the *P. gingivalis* group compared with the control group. However, the effects have been examined at 6 h and 48 h. Wang et al. asserted that there is a positive correlation between the expression of TNF-α and TLR4 in gingival fibroblasts surface (37). Also, the production of inflammatory cytokines IL-1 and IL-6 are triggered by TLR4 signaling in gingival fibroblasts, which can induce osteoclasts, resulting in alveolar bone resorption (38). Additionally, the high-level mRNA expression of TLR4, TLR7, and CD14 in healthy and periodontitis culture groups of periodontal cells was observed by Scheres et al. (39). Durand et al. reported that the concentrations of *E. coli* LPS 1, 5, and 10 µg/mL stimulated IL-6 production from MSCs after 72 h but had no effect on altered IL-18 expression (40). In the present study, only at 6 h, we observe an increase in TLR4 and IL-18 expression following the effect of *E. coli* LPS, the next time, the increased expression of TLR4 and IL-18 was not observed. However, IL-6 expression in the control group had no significant difference at any time point. IL-18 showed significant similarities between the results of the two studies, but the difference in the results of IL-6 may be correlated to the studied cell line. Bindal et al. reported that 1 and 2 µg/mL concentrations of LPS induce the expression of inflammatory cytokines like IL-6 and IL-8 in human dental pulp stem cells and can be considered a dental pulp microenvironment in an inflammatory condition (41). We found significant inflammatory effects in our study of the *P. gingivitis* LPS at a concentration of 1µg/mL at 24 h and then 6 h. Hashemzadeh et al. stated that TLR4 and IL-6 expression in mesenchymal stem cells of bone marrow increased after exposure by 1 and 10 µg/mL concentrations of S. flexneri LPS at 24 h, but the case of *S. dysenteriae* and *S. sonnei* was not affected (42). In the present study, we also observed increased expression of TLR4 and IL-6 after 24 h of the *P. gingivalis* LPS treatment, but there was no effect on the *E. coli* LPS. Taheri et al. reported that stimulation with LPS after 6 h did not affect IL-6 and IL-10 expression in human amniotic epithelial cells (43). In terms of IL-6 expression, after 6 h no tangible change has been observed in the current study. In the case of IL-10, the highest expression is seen in the LPS-affected groups. The reason for the discrepancy between the results could be related to the type of cell line studied, but it should be noted that in Taheri's study, LPS was used at a concentration five times that of the present study, which may have cytotoxic effects. It is also unclear which LPS was used by Taheri et al. Zhou et al. reported that LPS related to the *P. gingivalis* dose-dependency increased TLR4 expression after one hour. They showed no change in IL-6 expression after 24 and 48 h of LPS

stimulation (44). Despite studying different cell lines, the effect of LPS after 1 hour was not investigated. In this study, after 6 h, higher and more significant expression of TLR4 in the *P. gingivalis* group was observed, which confirms the similarity between these parts of both pieces of research. There was no statistically significant difference in IL-6 expression after 24 and 48 h following the *P. gingivalis* LPS effect. Yücel et al. reported that a 6-hour treatment with a concentration of 1 µg/mL of E. coli LPS increased inflammatory cytokines, but a 48-hour treatment increased the levels of anti-inflammatory cytokines such as IL-10 in cardiomyocyte-derived stem cells (45). In the present study, we found that after six h of stimulation with LPS related to E. coli, the expression of IL-18 as an important inflammatory cytokine increased. After 48 h stimulation, an increase in IL-10 expression was also observed, despite the differences in the cell line studied, confirming the high similarity between the findings. We also found a direct statistical correlation between IL-18 and TLR4 expression following the effect of *P. gingivalis* LPS, which was contrary to our study hypothesis, as we expected the inflammatory effects of the *P. gingivalis* LPS to be predominantly applied via TLR2, not TLR4, whereas we did not find a statistically significant correlation between TLR2 and inflammatory cytokine gene expression. In the case of *E. coli*, except for TLR2 expression, we found a statistically significant correlation between the expression of TLR4 and the expression of the studied cytokines. No similar study was found to compare the findings of correlation and their validity and reliability.

CONCLUSION

In sum, this study, and based on its findings, indicated that the effects of both types of LPS on inflammation could be mediated through TLR4. The *P. gingivalis* is more potent than *E. coli* in terms of arousing inflammation in the first 24 h, but after this time, not only does it act as an inflammatory stimulant but also by increasing IL-10 expression and significantly decreasing TLR4 expression and IL-18 probably provides the conditions for the differentiation of stem cells into the osteogenic/odontogenic line more favorably. Therefore, the use of the *P. gingivalis* LPS instead of the *E. coli* may seem to be more rational if we consider its time-dependent effects as it acts up to 24 h.

DECLARATION OF COMPETING INTEREST

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Conflict of Interest: None declared.

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