Expression Profiles of TGF-β and TLR Pathways in *Porphyromonas gingivalis* and *Prevotella intermedia* Challenged Osteoblasts

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Background: The presence of certain oral pathogens at implant sites can hinder the osseointegration process. However, it is unclear how and by what microorganisms it happens.

Objectives: This study investigated whether the presence of oral pathogens of *Porphyromonas gingivalis* and *Prevotella intermedia* individually, play a role in the failure of bone formation by determining the expression profiles of Transforming Growth Factor Beta (TGF-β/Bone Morphogenic Protein (BMP) and Toll-Like Receptor (TLR) pathways in challenged osteoblasts.

Materials and Methods: Cell viability of *P. gingivalis* and *P. intermedia* challenged osteoblasts were determined by WST assay. Changes in osteoblast morphology and inhibition of mineralization were observed by Scanning Electron Microscopy (SEM) and Von Kossa staining, respectively. Expression of TGF-β and TLR pathway genes on challenged cells were identified by RT profiler array. Both *P. gingivalis* and *P. intermedia* challenges resulted in reduced viability and mineralization of osteoblasts.

Results: Viability was reduced to 56.8% (*P. gingivalis*) and 52.75% (*P. intermedia*) at 1000 multiplicity. Amongst 48 genes examined, expressions of *BMPER, SMAD1, IL8* and *NFRKB* were found to be highly upregulated by both bacterial challenges (Fold Change > 4).

Conclusions: *P. gingivalis* and *P. intermedia* could play a role in implant failure by changing the expression profiles of genes related to bone formation and resorption.

Keywords: Receptors Transforming Growth Factor; Receptors, Toll Like; Bone Formation; Osseointegration

1. Background

Dental implants are a reliable oral and maxillofacial rehabilitation treatment modality. Despite high success rate, implant fixture failure may occur and approximately 2% of this failures have been associated with the failure in osseointegration (1). Osteoblasts play a crucial role for establishing or maintaining osseointegration since they directly differentiate from mesenchymal stem cells and produce the characteristic extracellular matrix that includes collagenous and non-collagenous proteins, Bone Morphogenetic Proteins (BMP), and growth factors (required for bone mineralization) (2). Osseointegration may be inhibited by bacterial or viral infections, which trigger the inflammatory response via cytokine regulation, thus decreasing the synthesis of the extracellular matrix (3). Implant failure cannot be related to a specific microorganism, but certain bacteria are present more frequently around failing implants, including *Porphyromonas gingivalis* and *Prevotella intermedia* (4). These are black pigmented, Gram negative, anaerobic pathogens that have multiple virulence factors such as lipopolysaccharide (LPS) and gingipains, which facilitate the adhesion and invasion of cells (5, 6).

Interactions between the pathogen and host cells lead to the degradation of the extracellular matrix and activation of gene expression; however, the genetic profiles of infected cells differ among various microorganisms (7). In this respect, pro-inflammatory and regulatory cytokines are key elements in determining disease progression. Of these cytokines, Transforming Growth Factor Beta (TGF-β) and Toll-Like Receptor (TLR) pathways related genes are critical elements in the control of tissue destruction and regeneration. Various genes of these pathways have previously shown links to creating an immune response to microbes, bone formation, and resorption (8-10). *Porphyromonas. gingivalis* is capable of interrupting the host immune system by inducing human peripheral macrophages and neutrophils to overproduce several proinflammatory cytokines such as interleukin-1 (IL-l), IL6, and Tumor Necrosis Factor *Alpha* (TNFα) (11). Also, *P. intermedia* induces interleukin-8 (IL-8) and mitogen activated protein kinases in monocytes, macrophages, endothelial cells, and gingival fibroblasts (12-14).

Other genes believed to be involved in bone formation are alkaline-phosphatase (*ALP*) and Biglycan (*BGN*) (15).

ALP is one of the main constitutions of bone extracellular matrix and has been used as a marker to determine bone formation (16). Another gene known to play a role in regulating bone formation and matrix mineralization is *BGN,* a member of the Small Leucine-Rich Proteoglycan (SLRP) family found in bone extracellular matrix. *BGN* modulates osteoblast differentiation, possibly by regulating BMP signaling and consequently matrix mineralization (15).

2. Objectives

This study focuses on determining the effects of oral pathogens; *P. gingivalis* and *P. intermedia*, on cell viability, matrix mineralization, and expression profiles of proinflammatory mediators and cytokine receptors in TGF-β and TLR pathways as markers for implant failure.

3. Materials and Methods

3.1. Bacterial Strains and Culture Conditions

Prevotella. intermedia (ATCC 25611) and *P. gingivalis* (ATCC 33277) cultures were obtained from the American Type Culture Collection (ATCC, USA). Microorganisms were cultured on Brain Heart Infusion Agar (BHIA) supplemented with 5% sheep blood (Salubris, Turkey) and incubated in an anaerobic workstation (Don Whitley, UK) at 37°C, in an atmosphere of 80% N_2, 10% H_2 and 10% CO_2 (HABAS, Turkey) for 3 - 4 d. Cultures were then maintained by sub-culturing every 2 - 3 d.

3.2. Cell Culture and Bacterial Challenge of the Human Fetal Osteoblast Cell Line

The Human Fetal Osteoblast Cell Line (hFOB 1.19) was obtained from the American Type Cell Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's Modified Eagle's Nutrient Mixture F-12 (DMEM F-12) (Invitrogen, Germany) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Invitrogen, Germany), 100 U/ mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Germany) in a 5% CO_2 incubator (Nuaire NU5510/E/G, USA) at 37°C. Human FOB cells were seeded at a density of 3 x 10^4 cell/cm^2 and allowed to attach overnight. Bacteria in the exponential phase of growth were washed and resuspended in the antibiotic-free DMEM-F12 at concentrations equivalent to multiplicities of infections (MOI, bacteria: cell ratio) of 100, 250, 500, and 1000. The MOIs of the inoculums were confirmed by plating serial 10-fold dilutions of *P. gingivalis* and *P. intermedia* anaerobically on BHIA and calculating the Colony Forming Units (CFU's) following incubation. Cells were then cultured in the presence or absence of *P. gingivalis* and *P. intermedia* at different multiplicities of infections for 2 hours in a humidified atmosphere of 5% CO_2 at 37°C. Experiments were carried out in triplicate cultures and 3 independent experiments were performed.

3.3. WST Assay

The effects of *P. gingivalis* and or *P. intermedia* challenge to viability of hFOBs were determined by measuring the mitochondrial dehydrogenase activity using the commercially available WST assay (Roche, Germany). Briefly, hFOB cells were challenged with *P. gingivalis* and *P. intermedia* at MOI of 100, 250, 500, and 1000 for 2 hours. Cells were then washed 3 times with Phosphate Buffer Saline (PBS) (Invitrogen, Germany) and incubated with DMEM F-12 supplemented with 200 μg/mL of metronidazole (Sigma-Aldrich, Germany), 300 μg/mL of gentamicin (Sigma-Aldrich, Germany) for 2 hours in 5% CO_2 at 37°C in order to kill any extracellular bacteria. A WST reagent was then applied to hFOBs according to the manufacturer's instructions. The absorbance was measured at 450 nm by a spectrophotometric plate reader (Bio-Tek ELx800, Germany). The values obtained represented the mean percentage (± SD) from 3 independent experiments.

3.4. Scanning Electron Microscopy

Monolayers of hFOBs were grown on 13-mm-diameter sterile plastic cell-culture coverslips (Nalge Nunc, NY USA) and were individually challenged with either *P. gingivalis* or *P. intermedia* at 1000 MOI. Following incubation, monolayers were washed with 1 mL of PBS (pH 7.2) and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (Sigma-Aldrich, Germany) pH 7.2, for 2 hours at 4°C. The samples were then washed 3 times in sodium cacodylate buffer (Sigma-Aldrich, Germany) and further dehydrated in a graded series of ethanol (Sigma-Aldrich, Germany) at room temperature and dried at critical point. Dehydrated samples were gold sputtered (Bal-Tec, Liechtenstein) for 5 minutes and examined by a 247 Zeiss evo40 SEM (Zeiss, Carl Zeiss SMT, Germany).

3.5. Assessment of Matrix Mineralization

Matrix mineralization of osteoblasts was assessed by using the modified Von Kossa silver nitrate staining method (17). Briefly, hFOB cells were cultured in the presence or absence of either *P. gingivalis* or *P. intermedia* at 1000 MOI. Then, hFOB cells were fixed in 2% paraformaldehyde (Sigma-Aldrich, Germany) for 30 minutes at 4°C. Following rinsing with PBS, the fixed plates were incubated with freshly prepared 3% silver nitrate solution (Merck, Germany) and exposed to UV light for 1 hour. Unreacted silver was then removed by incubation with 5% sodium thiosulphate (Merck, Germany) for 5 minutes and rinsed with distilled water. The cultures were then visualized by an inverted light microscope (Nikon Eclipse TS100, Japan) at 40× magnification.

3.6. RNA Extraction and cDNA Synthesis

Following bacterial challenge (MOI 1000), the culture supernatants were removed from the culture and the cell monolayers were washed twice in PBS before being lysed.

Total RNA was extracted from the collected cell lysate using the RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. RNA concentrations were measured using a NanoDrop spectrophotometer (NanoImplen, Germany). One microgram of total RNA was then reverse transcribed into single-stranded cDNA by using RT² First Strand Kit (SABiosciences, Qiagen, Germany) according to the manufacturer's instructions.

3.7. Real-Time Polymerase Chain Reaction Profiler Array

Gene expressions of TGF-β and TLR pathway were determined with RT profiler human Toll-Like receptor signaling pathway and TGF-β or BMP signaling pathway PCR array (SABiosciences, PAHS-035, and PAHS-018, respectively). A mixture of 1 μg of cDNA, 1350 μL RT-PCR SYBR green (Qiagen, Germany), and 1248 μL RNase free water was prepared and 25 μL of it was aliquoted into each well. Amplification was performed in a fluorescence thermocycler (Bio-Rad Laboratories, Germany) under the following conditions: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, finally annealing at 60°C for 1 minute. The specificity of PCR product was verified by melting curve analysis. The results obtained from PCR arrays were analyzed using a computer program RT Profiler PCR Array Data Analysis version 3.5 available at the company website (http://www.sabiosciences.com).

3.8. Quantitative Real Time Polymerase Chain Reaction

BGN and *ALP* genes, related to matrix mineralization and osseointegration were manually studied. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous RNA control in the samples (Housekeeping gene, HKG). qPCR was performed using 2.0 μL of cDNA in a 25 μL SYBR green reaction mix (Qiagen, Germany) for 15 minutes at 95°C and 40 cycles of 10 seconds at 95°C, annealing at 58°C for 1 minute. The specificity of PCR product was verified by melting curve analysis. The relative expressions compared to the HKG were calculated by the comparative Ct method (2 ΔCt formula) following normalization to *GAPDH*. The primers were used as follows: *GAPDH* 5'-TCCATGACAACTTTGGTATCG-3', 5' TGTAGC-CAAATTCGTTGTCA-3', (18), Alkaline phosphatase (*ALP*) 5'-CTTGACTGTGGTTACTGCTG-3'; *ALP*, 5'-GAGCGTAATCTAC-CATGGAG-3'., (19), Biglycan (*BGN*), 5'-CTCAACTACCTGC-GCATCTCAG-3', 5'-GATGGCCTGGATTTTGTTGTG-3' (*ALP*ha DNA, Canada) (20).

4. Results

Cell viability of challenged hFOB cells showed a marked decrease (in a dose-dependent manner) in cell viability following both *P. gingivalis* and *P. intermedia* challenges. At the highest MOI of 1000, the viability was observed to reduce to 56.8% (*P. gingivalis*) and 52.75% (*P. intermedia*). No significant difference was observed between the two species at all MOI ($P > 0.05$). The effects of bacterial challenge on osteoblast morphology were observed by SEM. Osteoblasts with a normal flattened fibroblast-like morphology were seen to detach and change to a more spherical one in *P. gingivalis* challenged cells (Figure 1), suggesting cell death. Similar results were observed for *P. intermedia* challenged osteoblasts. The negative effects of both *P. gingivalis* and *P. intermedia* were verified by a large reduction in mineralization of the osteoblasts.

The panel of cytokine genes and receptor pathways were selected (Tables 1 and 2), either according to their association with bone morphogenesis or because they are genes coding for important inflammation-associated molecules that may be involved in the bacterial challenge process. Array results confirmed the detrimental effect of the presence of these oral pathogens on bone formation. Overall, the BMP receptors (BMPRs) and activin receptors (ACVRs) were seen to be highly upregulated, with bone morphogenetic protein receptor, type IB (BMPR1B) showing the highest fold increase following *P. gingivalis* challenge and BMP binding Endothelial Regulator (BMPER) for *P. intermedia* challenged cells. The direction of expression was differentially regulated in Collagen, type I, *ALP*ha 1 (*COL1A1*), Collagen, type I, *ALP*ha 2 (*COL1A2*), osteocalcin (*OC*), and Bone Morphogenetic Protein Receptor, type IA (*BMPR1A*) for *P. gingivalis* and *P. intermedia*. A downregulation was observed for *P. gingivalis* infected cells in comparison to an upregulation observed following *P. intermedia* challenge. A reduction in matrix mineralization was observed in *P. gingivalis* challenged cells following Von Kossa staining. Conversely, an upregulation was observed in *P. intermedia* challenged cells for *COL1, OC*, and *BMPR1A*.
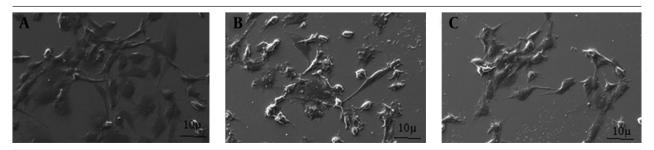

Figure 1. Scanning Electron Microscopy Images of Challenged Osteoblasts by *Porphyromonas gingivalis* and *Prevotella intermedia* at a MOI of 1000 for 2 Hours

Table 1. Fold Change of TGF Pathway Genes in hFOB Cells Following Challenge by *Porphyromonas gingivalis* or *Prevotella intermedia*

Gene Description	Gene Symbol	Accession No [a]	Fold Change	
			Porphyromonas gingivalis[b]	*Prevotella intermedia*[c]
Activin A receptor, type I	*ACVR2A*	NM-001616	3.34	6.681
Osteocalcin	*OC*	NM-199173	0.895	3.58
BMP binding endothelial regulator	*BMPER*	NM-133468	5.816	23.264
Bone morphogenetic protein receptor, type IA	*BMPR1A*	NM-004329	0.551	7.674
Bone morphogenetic protein receptor, type IB	*BMPR1B*	NM-001203	40.504	3.58
Bone morphogenetic protein receptor, type II	*BMPR2*	NM-001204	14.32	5.816
Collagen, type I, alpha 1	*COL1A1*	NM-000088	0.24	13.361
Collagen, type I, alpha 2	*COL1A2*	NM-000089	0.008	6.233
Collagen, type III, alpha 1	*COL3A1*	NM-000090	1.919	3.58
SMAD family member 1	*Smad1*	NM-005900	4.113	15.348
SMAD family member 2	*Smad2*	NM-005901	0.895	3.117
SMAD family member 3	*Smad3*	NM-005902	0.004	0.678
SMAD family member 4	*Smad4*	NM-005359	0.003	4.724
SMAD family member 5	*Smad5*	NM-005903	0.016	0.148
SMAD specific E3 ubiquitin protein ligase 1	*SMURF1*	NM-020429	0.678	10.126
Signal transducer and activator of transcription 1, 91kDa	*STAT1*	NM-007315	0.002	6.681
Runt-relatedtranscription factor 1	*RUNX1*	NM-001754	1.454	11.632
Serpin peptidase inhibitor, clade E, member 1	*SERPINE1*	NM-000602	0.021	10.126
Transforming growth factor, beta-induced, 68kDa	*TGFB1*	NM-000660	0.042	6.233
Transforming growth factor, beta receptor 1	*TGFBR1*	NM-004612	3.58	1.454
Transforming growth factor, beta receptor 2	*TGFBR2*	NM-003242	0.097	0.182
Transforming growth factor, beta receptor 3	*TGFBR3*	NM-003243	3.117	0.514
TGFB-induced factor homeobox 1	*TGIF1*	NM-003244	0.097	0.633
Latent transforming growth factor beta binding protein 1	*LTBP1*	NM-000627	0.678	2.713
Latent transforming growth factor beta binding protein 2	*LTBP2*	NM-000428	3.117	2.908
Latent transforming growth factor beta binding protein 4	*LTBP4*	NM-003573	1.028	0.418

[a] Fold change value of a gene following *P. gingivalis* challenge.
[b] Fold change value of a gene following *P. intermedia* challenge. Fold change of mRNA level was calculated relative to the expression value in non-challenged cells. Fold-change values less than one indicate a negative or down-regulation
[c] Accession numbers indicate the sequence used as Super Array analysis.

Table 2. Fold Change of TLR Pathway Genes in hFOB Cells Following Challenge by *Porphyromonas gingivalis* or *Prevotella intermedia*

Gene Description	Gene Symbol	Accession No [a]	Fold Change	
			Porphyromonas gingivalis[b]	*Prevotella intermedia*[c]
Toll-like receptor 3	*TLR3*	NM-003265	0.025	0.021
Toll-like receptor 4	TLR4	NM-138554	1.231	2.144
Toll-like receptor 5	TLR5	NM-003268	0.002	0.00
Toll-like receptor 6	TLR6	NM-006068	0.002	0.005
Myeloid differentiation primary response gene (88)	MYD88	NM-002468	1.741	3.482
Toll interacting protein	TOLLIP	NM-019009	3.732	1.516
Toll-like receptor adaptor molecule 1	TICAM1	NM-182919	1.414	1.072
Toll-like receptor adaptor molecule 2	TICAM2	NM-021649	0.095	0.354
Interleukin 12A	IL12A	NM-000882	0.009	0.006
Interleukin 1A	IL1A	NM-000575	0.467	0.379
Interleukin 1B	IL1B	NM-000576	0.536	0.536
Interleukin 6	IL6	NM-000600	0.354	0.083
Interleukin 8	IL8	NM-000584	17.148	12.126
Interleukin-1 receptor-associated kinase 1	IRAK1	NM-001569	0.095	0.144
Interleukin-1 receptor-associated kinase 2	IRAK2	NM-001570	3.031	5.657
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFKB1	NM-003998	2	1.866
NFKB inhibitor, alpha	NFKBIA	NM-003998	0.707	0,33
Nuclear factor related to kappaB binding protein	NFRKB	NM-006165	6.498	21.112
Mitogen-activated protein kinase kinase 3	MAP2K3	NM-002756	0.203	0.095
Mitogen-activated protein kinase kinase 4	MAP2K4	NM-003010	0.117	0.154
Mitogen-activated protein kinase kinase kinase 1	MAP3K1	NM-005921	0.014	0.016
Mitogen-activated protein kinase kinase kinase kinase 4	MAP4K4	NM-004834	1.32	1

[a] Accession numbers indicate the sequence used as Super Array analysis.
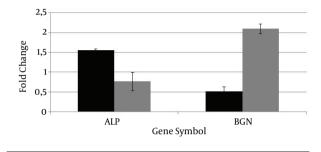[b] Fold change value of a gene following *P. gingivalis* challenge.
[c] Fold change value of a gene following *P. intermedia* challenge. Fold change of mRNA level was calculated relative to the expression value in non-challenged cells. Fold-change values less than one indicate a negative or downregulation.

Smad genes were also examined. *P. gingivalis* displayed an overall marked downregulation of these genes except in *Smad* family member 1 (*Smad*1). On the other hand, *P. intermedia* showed high upregulation of all the *Smad* family genes with the exception of *Smad* family member 3 (*Smad*3) and *Smad* family member 5 (*Smad*5). In this study, TGF-β receptors were studied for their relation to bone formation. In general *P. gingivalis* challenged cells were downregulated. Transforming Growth Factor Beta (*TGFB1*) and Transforming Growth Factor Beta Receptor (*TGFBR3*), were seen to be highly expressed in *P. intermedia*

and *P. gingivalis*, respectively. Furthermore, an increase in gene expression of Toll-like Receptor 4 (TLR4), *MYD88*, Interleukin-1 Receptor-Associated Kinase 2 (*IRAK2*) and *NF-κB* were observed. On the otherhand, the expressions of *TLR*s 3, 5, and 6 and all interleukins with the exception of *IL*8, were downregulated by both bacterial challenges.

The levels of Mitogen-Activated Protein Kinase Kinase 3 (*MAP2K3*), Mitogen-Activated Protein Kinase Kinase 4 (*MAP2K4*) and Mitogen-Activated Protein Kinase Kinase 1 (*MAP3K1*) expression were decreased by *P. gingivalis* and *P. intermedia* challenge. Conversely, expression

of Mitogen-Activated Protein Kinase Kinase Kinase Kinase 4 (*MAP4K4*) showed an upregulation by *P. gingivalis* challenge. In addition, *ALP* and *BGN* were also studied by manual qPCR. Fold regulations of these genes are shown in Figure 2. Expression of *ALP* was seen to increase in *P. gingivalis* challenge, and decrease in *P. intermedia* challenge. The opposite was observed for *BGN* expression levels.

Figure 2. Relative Gene Expression (Fold Change) was Determined by Real-Time PCR Using Specific Primers to *ALP* and *BGN* Genes Following Bacterial Challenges

The gene expression of *Porphyromonas gingivalis* challenged osteoblasts is shown as black bars. The gene expression of *Prevotella intermedia* challenged osteoblasts is shown as grey bars.

5. Discussion

In this study no significant difference was observed between the *P. gingivalis* and *P. intermedia* MOI (P > 0.05). These findings were similar to those of Kadono et al. (13), who found that *P. gingivalis* cell invasion adversely affected osteoblasts differentiation and activity. Although a previous study by Zhang et al. showed that an increase in bacterial concentration gave way to more bacterial invasion of the cell, this did not affect cell viability or proliferation (21). The difference between these results could be due to different methods used for measuring cell viability. SEM results showed a large reduction in mineralization of the osteoblasts. These findings have previously been observed in mouse calvarial osteoblasts following *P. gingivalis* invasion (21). Similar observations have been documented for *P. intermedia* challenged cells where a reduction in alkaline phosphatase activity and calcium incorporation were seen (22).

It has been previously shown that upregulation of *BMPER* inhibits bone morphogenetic protein 2 (BMP2) and bone morphogenetic protein 4 (BMP4), dependent osteoblast differentiation and BMP-dependent differentiation of the chondrogenic cells (23). The *BMPR-1B* gene has been suggested as a therapeutic target for enhancing bone regeneration in vivo (24). The converse upregulation following bacterial challenge could suggest a repairing mechanism of this gene in order to inhibit negative effects of bacterial presence. These genes are known to play role in matrix mineralization (25, 26).

Collagen, the most abundant organic component of bone has a structural function. Type I and III collagens have been implicated as regulatory molecules for the differentiation and proliferation of human osteoblastic cells, and as a possible new target for the treatment of osteoporosis, although collagen III is found at very low level in the bone matrix (27). The reduction in matrix mineralization observed in *P. gingivalis* challenged cells following Von Kossa staining is in accordance with the downregulation of *COL1A1* and *COL1A2*. However, an upregulation was observed in *P. intermedia* challenged cells for *COL1, OC*, and *BMPR1A*. It has previously been suggested that deletion of *OC* increases bone mass (28). These differences in gene expressions suggest that *P. gingivalis* and *P. intermedia* might have different mechanisms in inhibiting matrix mineralization of osteoblasts.

Genes from the mothers against decapentaplegic homolog (*Smad* family) were also examined. A marked difference in the expression of *P. gingivalis* and *P. intermedia* infected cells was observed. *P. gingivalis* displayed an overall marked downregulation of these genes except in *Smad* family member 1 (*Smad*1). On the other hand, *P. intermedia* showed high upregulation of all the *Smad* family genes with the exception of *Smad* family member 3 (*Smad*3) and *Smad* family member 5 (*Smad*5). The *Smad* gene family provides instructions for producing proteins that help regulate the activity of particular genes as well as cell growth and proliferation (29). The downregulation of *Smad*3 and *Smad*5 by both *P. gingivalis* and *P. intermedia* is in accordance with findings that showed a deletion of these genes lower the rate of bone formation and mineralization (30). The dowregulation could be due to positively regulated *BMPER* since it is known that *BMPER* antagonizes *BMP4*-dependent *Smad*5 activation (23).

In this study, TGF-β receptors were also studied for their relation to bone formation. In general *P. gingivalis* challenges cells showed downregulation. A study by Filvarof et al. showed that inhibition of TGF-β receptor signaling in osteoblasts lead to decreased bone remodeling (31). This study confirmed these findings for both *P. gingivalis* and *P. intermedia* but was differential in the expressions of Transforming Growth Factor Beta-induced (*TGFB1*) and Transforming Growth Factor Beta Receptor (*TGFBR3*), which were seen to be highly expressed in *P. intermedia* and *P. gingivalis*, respectively.

In the TLR pathway, genes related to toll-like receptor, Interleukin 1 (*IL*), Mitogen-Activated Protein Kinase (*MAP*), and Nuclear Factor-kappa-B (*NF-κB*) family were examined. All these pathways are known to play a role in bone regeneration and cell cycle (32-34). The expression profiles for TLRs were consistant with their known function of being pattern recognition receptors that recognize foreign substances in the body and activate immune system (35). Such an increase in gene expression of Toll-like Receptor 4 (TLR4) in human periodontal ligament cells challenged by *P. intermedia* has previously been observed (36). Sartori et al. (37) indicated that TLR4 signaling activates Myeloid Differentiation Primary Response gene 88 (*MYD88*) dependent pathways to subse-

quent activation of Interleukin-1 Receptor-Associated Kinase (*IRAK*), TNF Receptor-Associated Factor 6 (*TRAF6*), and, ultimately *NF-κB*, which is required for cytokine induction (37). Upregulation of *MYD88*, Interleukin-1 Receptor-Associated Kinase 2 (*IRAK2*) and *NF-κB* observed in this study is consistent with these findings. Similar findings have been reported in a study were an increase in the expression of *MYD88* was found following Salmonella infection (38).

Our findings suggest that *P. gingivalis* and *P. intermedia* are likely to act via *TLR*4. The suppressed expressions of *TLR*s 3, 5, and 6 could be due to upregulation of Toll Interacting Protein (TOLLIP), an inhibitory adaptor protein which was also upregulated in this study (39). The *IL* family plays a central role in the regulation of immune and inflammatory responses. Interestingly the expressions of all interleukins apart from *IL*8 were downregulated by both bacterial challenges. Similar finding have shown *IL*8 to be upregulated by oxidative stress (40). Such an upregulation might be indicative of osteoblast infection by *P. gingivalis* and *P. intermedia*. The *MAP* family plays an important function in regulating the proinflammatory cytokines (41). Riewe et al. showed that *P. gingivalis* infection induced phosphorylation and activation of *MAPK2K3* in human extravillous trophoblasts (42). Although there are not many studies on *MAP3K1*, an overexpression of *MAP3K1* has previously been found in extramammary Paget's disease (43). Different results could be due to the different cell line and strains used. Interestingly, expression of *MAP4K4* did not change in *P. intermedia* challenged cells.

The reduced expression of *ALP* could be related to the reduction in mineralization in *P. intermedia* challenged cells. Furthermore, the reduction in mineralization of osteoblasts by *P. gingivalis* challenge could be due to decreased expression of *BGN*. It was shown that *P. gingivalis* LPS's significantly delay normally high expression levels of *BGN* in rat alveolar bone osteoblasts (44).

In conclusion, the results of this study showed that both *P. gingivalis* and *P. intermedia* were capable of inhibiting osteoblast proliferation and therefore osseointegration. Both of these anaerobes were also seen to reduce matrix mineralization in osteoblast cells. Moreover, gene expression studies showed that *P. gingivalis* and *P. intermedia* induced TLR and TGF beta related genes, by upregulation or downregulation of their cytokines and receptors. Furthermore, differences were observed in the expression of several genes following *P. gingivalis* and *P. intermedia* challenge. These differences suggest that *P. gingivalis* and *P. intermedia* could have different mechanisms of inhibiting or reducing bone formation. The changes observed in the expression of key genes involved in bone resorption and formation indicates an important role for these bacteria in reducing osseointegration. The findings of this study confirm that both *P. gingivalis* and *P. intermedia* are risk factors for failure in bone formation and bone resorption.

Authors' Contributions

May Korachi: Study concept and design, drafting of the manuscript; Fatma Yesim Ekinci: Analysis and interpretation of data; Kubra Aydin: Performed all experiments.

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