ORIGINAL RESEARCH

Differential Expression of Toll-like Receptor 4 and Nuclear Factor κB of Primary Rat Oral Keratinocytes in Response to Stimulation with *Fusobacterium nucleatum*

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ABSTRACT

Aim: Gram-negative anaerobic periodontal pathogens are well known for their ability to stimulate innate immune response once being recognized by special receptors of epithelial pocket lining. The aim of this study was to investigate the expression of toll-like receptor 4 (TLR4) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) following exposure to *Fusobacterium nucleatum*.

Materials and methods: Primary oral keratinocytes were collected from gingiva of Wistar Han rat and cultured to confluency. Then, the epithelial cells were stimulated with heat-killed *F. nucleatum* and 20 μg/mL *Escherichia coli* lipopolysaccharide (*E. coli* LPS). Expression of NF-κB was assayed by immunocytochemistry in addition to semi-quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR), which was also used to determine transcription of TLR4 in comparison with unstimulated control.

Results: Immunocytochemical analysis indicated that nuclear translocation of NF-κB was significantly increased ($p < 0.05$) in cultures stimulated with bacteria as compared with control. In addition, the expression of TLR4 and NF-κB in stimulated keratinocytes was also significantly increased ($p < 0.05$) 3- and 2.5-fold respectively, in comparison with unstimulated cultures.

Conclusion: *Fusobacterium nucleatum* potentially triggers NF-κB signaling pathway through activation of TLR4 in oral epithelial cells.

Clinical significance: Recognition of early immune response against pathogenic periodontal bacteria would highlight new aspects in pathogenesis of periodontal diseases that could provide potential therapeutic opportunity for these conditions.

Keywords: *Fusobacterium nucleatum*, Gingival epithelium, Nuclear factor κB, Toll-like receptor 4.

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INTRODUCTION

Periodontitis is an inflammatory process affecting tooth-supporting apparatus. Undiagnosed and untreated periodontitis gradually leads to permanent periodontal tissue breakdown and eventually tooth loss. Such a condition is induced by oral microbial biofilm attached to tooth surfaces mainly subgingivally in deep pockets. At these sites, principal periodontal pathogens, such as *F. nucleatum*, can be found and were reported to have virulence potential strongly associated with the initiation and progression of periodontitis.[1] In susceptible patients to periodontitis, a series of aberrant host responses are developed in response to pathogens, mainly Gram-negative anaerobic bacteria.[2] *Fusobacterium nucleatum* is one of these periodontal pathogens, which is well recognized by host epithelial cells of gingival crevice, resulting in secretion of a range of proinflammatory cytokines important in initiating the inflammation.[3]

Epithelial lining of gingival crevice protects the underlying tissue from invading pathogenic bacteria in subgingival sites as part of innate immunity.[4] Each cell expresses surface proteins receptors, such as TLR, which recognize bacteria and their antigenic cell parts, and thus induce innate immune responses.[5] To this date, over 11 human TLRs have been reported recognizing different bacterial cell parts, such as bacterial deoxyribonucleic acid (DNA), LPS, and lipoteichoic acid.[6] One of these receptors is TLR4, which is reported to be involved in recognition of bacterial LPS.[7] Gingival samples collected from extracted teeth at terminal stage of periodontitis showed upregulation of TLR4 expression when compared with healthy samples.[8] Previous studies indicated that signaling downstream of TLR4 leads to nuclear translocation of NF-κB,[9,10] which is a critical transcriptional factor, highly associated with upregulation of inflammatory cytokines, potentially involved in development and progression of periodontitis.[11,12] Interaction of epithelial pocket lining with pathogenic microflora elicits intense immune response, and immunohistochemical analysis of gingival samples collected from patients with periodontitis showed increased cytoplasmic and nuclear activity of NF-κB subunits p50 and p65 as compared with healthy controls.[12] Further, exposure of oral squamous cell carcinoma cell line of

epithelial origin to *F. nucleatum* for 1 hour was associated with increased NF-κB activity.[13] Similarly, coculturing *F. nucleatum* with two cell lines also induced significant increase in nuclear activity of NF-κB when compared with unstimulated control.[14]

Evidence from previous studies suggested that anaerobic periodontal pathogens and their virulence factors are responsible for triggering TLR4 signaling cascade and increased transcriptional activity of NF-κB. Thus, the current study was conducted to investigate the *in vitro* expression of TLR4 and subsequent NF-κB activation in rat oral keratinocytes following stimulation with *F. nucleatum*.

MATERIALS AND METHODS

Cell Culture and Bacterial Stimulation

This study was conducted in the Department of Periodontics, University of Baghdad. Total number of samples (*N*) included for each experiment was 36. Anaerobic culture and heat-killing protocol of *F. nucleatum* (American Type Culture Collection 10953™) (ATCC®, Rockville, Maryland, USA) was performed according to methods previously described.[13,15]

Labial gingival samples of Wistar Han male rat carcass were excised and immersed overnight at 4°C with 1 mL 0.25% trypsin. Epithelial sheet was separated using fine tweezer, then dissociated into smaller pieces, centrifuged at 800 rpm, then collected, and seeded in flask containing keratinocyte serum-free medium (Gibco BRL, New York, New York, USA) with 50 mg/mL of gentamycin.[16] Once growth of epithelial colonies was evident, oral keratinocytes were subcultured and used for different experiments.

Cell cultures were stimulated with 20 μg/mL *E. coli* LPS (positive control) and heat-killed *F. nucleatum*, and the ratio of bacteria to cell was 100 bacteria per cell similar to the ratio in periodontal pocket.[17] Negative control for all experiment was cultures treated with media only.

Immunocytochemistry

Oral keratinocytes were seeded (4 × 10^5 cells) on multi-well slides (Hedley-Essex, UK) placed in glass Petri dish containing 15 mL of media and incubated for 5 days until reaching 70 to 80% confluency. At the day of experiment, epithelial cultures were stimulated with heat-killed *F. nucleatum* and 20 μg/mL *E. coli* LPS for 1 hour, then cultures were washed with phosphate-buffered saline (PBS), and fixed with acetone for 15 minutes at room temperature. Then, 100 μL of monoclonal antibody to NF-κB p65 subunit (Santa Cruz Biotechnology, Santa Cruz, California, USA) at a dilution of 1:100 in PBS bovine serum albumin 1% was added and the slides were placed in a humidity box at room temperature for 1 hour. Staining of the target antigen was performed using SuperSensitive® Link-Detection Kit (BioGenex, Fremont, California, USA) according to the manufacturer's instruction. Slides were then washed with deionized water for 2 minutes, then dehydrated through graded alcohol baths, and cleared in xylene (Sigma, UK) before being mounted in XAM mounting medium. The slides were viewed under the microscope (Primovert, Zeiss, Germany) using 10× objective lens. Percent of positively stained nuclei (dark brown) was counted relative to total number of nuclei (positive and negative) viewed in the field. Five random fields were selected for each slide and the experiment was repeated in triplicate.

Semi-quantitative RT-PCR

Oral keratinocyte cultures were stimulated as previously described for 24 hours, then media was removed, and RLT lysis buffer was added to make cell lysate. This lysate was used to extract total ribonucleic acid (RNA) by using RNA extraction kit purchased from Qiagen, UK. Single-stranded complementary DNA was synthesized from 1 to 2 μg of DNase-digested total RNA using Tetro kit (Bioline, London, UK) following the manufacturer's manual.

For analysis, 1 μL of single-stranded complementary DNA template was added to RedTaq MasterMix (Sigma, UK) with selected primer. Details and primer (all supplied from Invitrogen, UK) sequences used for PCR assay are listed in Table 1. This mixture was amplified using a thermal cycler (Mastercycler, Eppendorf, Germany) in range of 18 to 35 cycles. Each cycle consists of 94°C for 20 seconds, 60 to 61°C for 20 seconds, and 72°C for 20 seconds ending with a 10-minute extension at 72°C. Samples were transferred to a 1.5% agarose gel containing 0·5 μg/mL ethidium bromide (Sigma, UK). Reaction

Table 1: Details of primers

| Gene name	Gene symbol	Accession number	Sequence (5'–3')	Tm (°C)	Product (bp)
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_017008	F-CGA TCC CGC TAA CAT CAA AT R-GGA TGC AGG GAT GAT GTT CT	60	597
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB	XM_219954	F-AACTCTGGGAAGCTGAACCG R-AAGCGTAGCCGTACAATGCT	60	641
Toll-like receptor-4	TLR4	NM_0191778.1	F-TTGAAGACAAGGCATGGCATGG R-TCTCCCAAG ATCAACCGATG	60	507
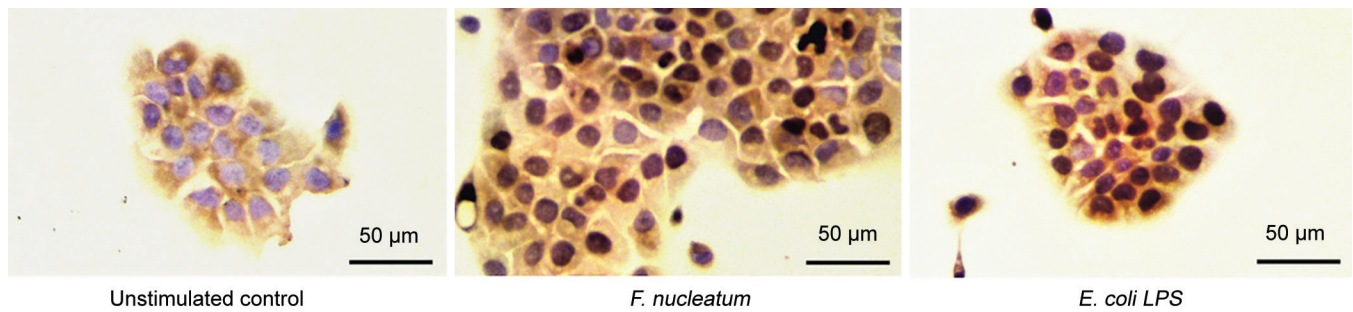
Fig. 1: Immunocytochemical images of oral epithelial cells stimulated with *F. nucleatum* and *E. coli* LPS show increased nuclear translocation of NF-κB (darkly stained nuclei) as compared with unstimulated control (violet clear nuclei)

products were separated by gel electrophoresis and visualized by scanning the gels in ultraviolet box. Pictures of gels were captured and analyzed by using GeneTools software (Syngene, Frederick, Maryland, USA). Data were exported to Microsoft Excel and normalized against glyceraldehyde-3-phosphate dehydrogenase. Experiment was run in triplicate.

RESULTS

### Activation of NF-κB in Response to Bacterial Stimulation

Immunocytochemical staining of rat oral epithelial cells treated with *F. nucleatum* and 20 μg/mL *E. coli* LPS showed increased nuclear activity of NF-κB. This was manifested by dark brown discoloration of nuclei and cytoplasm in cultures stimulated with bacteria *vs* cultures treated with media only that showed clear nuclei with 3,3'-diaminobenzidine stain concentrated in cytoplasm only (Fig. 1). Analysis of slides indicated significant upregulation ($p \leq 0.001$) in percent of positively stained nuclei in association with *F. nucleatum* (76%) and 20 μg/mL *E. coli* LPS (66%) when compared with unstimulated control (8%) (Graph 1).

Gene Expression of TLR4 and NF-κB

Exposure of rat oral keratinocytes to bacterial stimulation for 24 hours upregulated the transcription of TLR4 and NF-κB (Fig. 2A). Expression of TLR4 was increased following coculturing with *F. nucleatum* (3-fold) and *E. coli* LPS (4-fold) in comparison with unstimulated control (Fig. 2Bi). This was associated with upregulation of NF-κB expression in response to stimulation with bacteria. *Fusobacterium nucleatum* increased gene expression of this transcription factor up to 2.5-fold, while *E. coli* LPS upregulated its expression to 1.5-fold as compared with cultures treated with media only (Fig. 2Bii).

DISCUSSION

Oral epithelium acts as a physical barrier against bacteria in addition to immunological role by sensing the presence
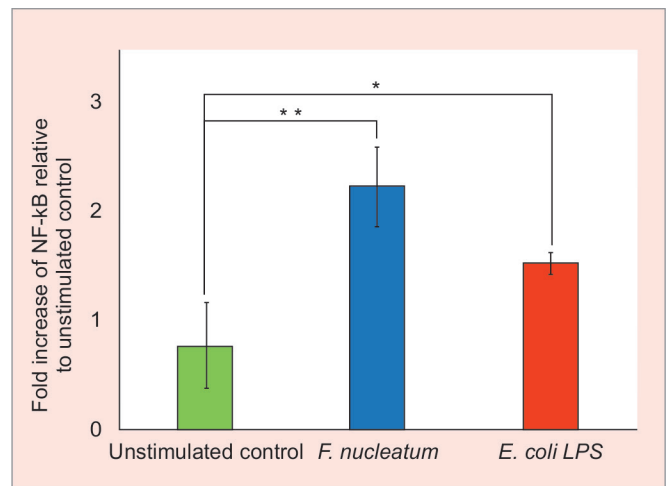
Graph 1: Analysis of immunocytochemical data indicates that the percent of cells with positive nuclear activity of NF-κB which was associated with *F. nucleatum* was significantly higher than unstimulated control (**$p \leq 0.001$)

of bacteria and subsequent secretion of inflammatory mediators that are considered as part of innate immune system.[4] This immune response depends largely on family members of pattern recognition receptors (PRRs) that recognize conserved structure of pathogens known as pathogen-associated molecular patterns.[5,18] The TLRs are well-recognized PRRs that detect certain antigenic molecules, such as TLR4 highly associated with LPS recognition.[7,11,19] Microenvironment of periodontal pocket harbors a wide range of pathogenic bacteria in addition to their virulence factors including LPS, which is a highly potent inflammatory stimulator. However, the severity of inflammatory response to LPS differs according to the bacterial origin of this pathogenic molecule.[20] Signaling downstream of TLR is well recognized in activation of transcriptional factor NF-κB[9,10] that was first described by Sen and Baltimore,[21] leading to increased production of proinflammatory cytokines critical for initial immune response during the course of periodontal disease.[12,13] Thus, the current study was designed to investigate *in vitro* the potential of *F. nucleatum* to trigger TLR4-mediated signaling together with transcriptional activity of NF-κB in culture of primary rat oral keratinocytes.
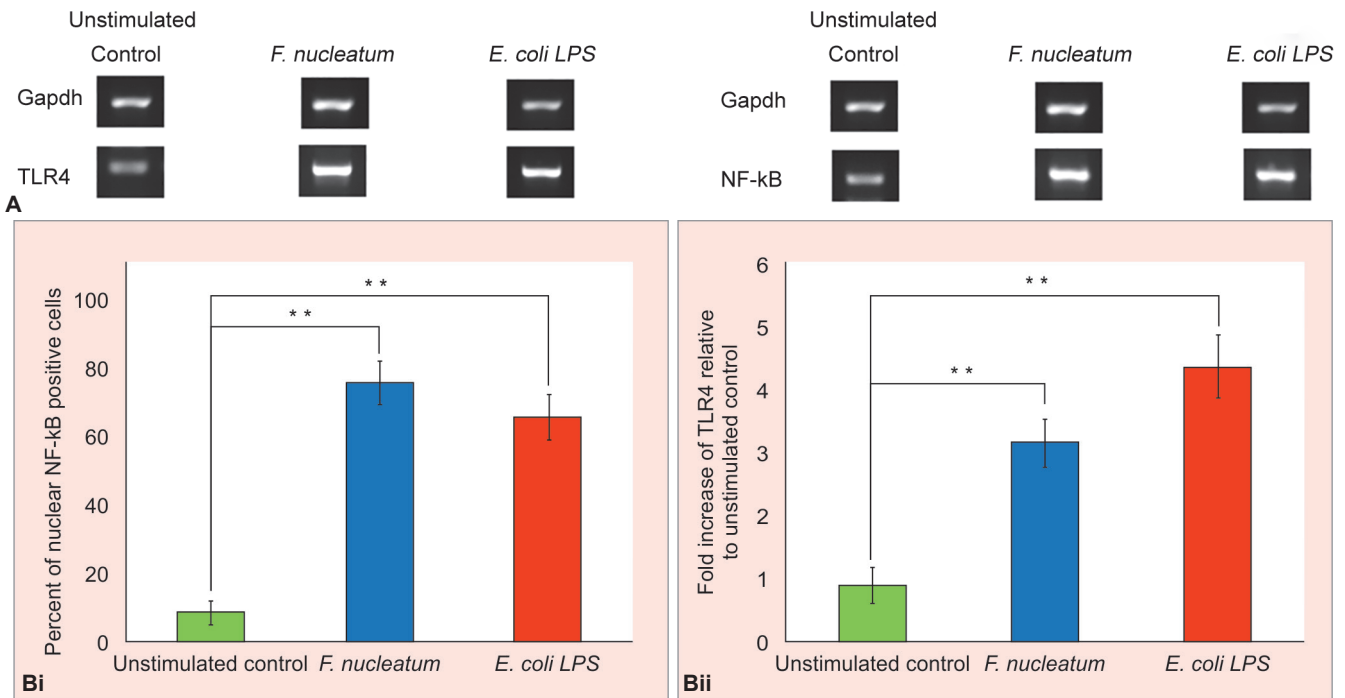
Figs 2A and B: Gel images (A) of TLR4 and NF-κB following a 24-hour exposure to bacterial components (*F. nucleatum* and *E. coli* LPS). Densitometric analysis (B) shows that TLR4 was upregulated 3- to 4-fold following stimulation with bacteria as compared with unstimulated control (i). Similarly, NF-κB increased 2.5-fold in association with *F. nucleatum* and 1.5-fold in response to *E. coli* LPS relative to cells treated with media only (ii) (*$p \leq 0.05$, **$p \leq 0.001$)

Fusobacterium nucleatum is one of the key periodontal pathogens highly involved in inducing immune response, changing pocket microenvironment, and cross-talking with other bacteria,[22] thereby favoring the growth of more pathogenic flora mainly dominated by red complex microorganisms.[2] Nevertheless, results from previous studies showed controversy about the ability of *F. nucleatum* to activate TLR4 and NF-κB downstream. Classical proinflammatory response typically induced by NF-κB pathway seems not involved by stimulation with *F. nucleatum* which utilizes alternative pathway (mitogen-activated protein kinase), instead to upregulate production of interleukin 8.[22] Further, treatment of human gingival epithelial cells with *F. nucleatum* for 24 hours resulted in suppression of NF-κB function.[23] Activation of NF-κB is known to be a consequence of TLR, highly expressed in oral epithelium signaling.[24] González et al[25] demonstrated that coculturing of *F. nucleatum* with monocytes/macrophages for 6 hours increased transcription of TLR2 and TLR9, while TLR4 did not show any significant change in comparison with control. In contrast, immunocytochemical analysis and PCR assay of H400 oral keratinocytes cultures, exposed to *F. nucleatum* and *P. gingivalis* over 24 hours, showed significant upregulation of TLR4 and NF-κB activity,[13] which agree with findings from our study. Treatment of human periodontal ligament cells with *E. coli* LPS resulted in upregulation of 12 inflammatory markers

including TLR4[11] which is also similar to results of current study. Consistently, *F. nucleatum* showed the ability to induce TLR4 activation in mice both *in vitro* and *in vivo* associated with increased inflammatory response that was ceased in TLR4-deficient mice.[26] In addition, gingival samples collected from patients with chronic periodontitis indicated increased expression of TLR4[8] and NF-κB[12] when compared with healthy controls. The differences in results of previously mentioned studies could be due to variations in experimental period, use of different strains of *F. nucleatum*, and different cell types that may differ in their response to the same stimuli under the same experimental conditions.

Limitations with the findings of current study may include lack of use of clinical bacterial strains, isolated from dental plaque samples, which could provide closer results to *in vivo* situation. In addition, epithelial cultures based on tissue samples collected from healthy and periodontally involved patients, rather than animal tissue, would also strengthen the findings. Furthermore, *F. nucleatum* can trigger different signaling downstream other than TLR4–NF-κB pathway possibly involved in inducing the immune response that requires further investigation.

CONCLUSION

Results of this study indicated that *F. nucleatum* could induce TLR4–NF-κB signaling downstream in primary

oral epithelial cells that may be responsible for initiation and progression of periodontal disease. However, further studies are needed to investigate the variable behavior of this oral commensal in immunomodulation in health and disease, which could provide potential therapeutic approach for treatment of periodontal disease.

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