The effects of *Enterococcus faecalis* on PMNL functions: an in vitro study

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SUMMARY

The aim of this study was to evaluate the effects of *E. faecalis* on chemotactic and oxidative burst activity of polimorphonuclear leukocytes (PMNLs) using transwell system and flow cytometry. PMNL cultures of six healthy donors were incubated with or without *E. faecalis* culture supernatants in order to determine the effects of this microorganism on PMNL functions. In addition, the effects of soluble factors derived from active mononuclear cells on the PMNL functions against *E. faecalis* were tested in culture conditions. Chemotactic and oxidative burst activity were significantly higher in PMNL cultures with *E. faecalis* culture supernatants alone or combined with stimulated mononuclear cell culture supernatants than those of PMNL cultures without *E. faecalis* and stimulated mononuclear cell culture supernatants. Oxidative burst activity in PMNL cultures combined with *E. faecalis* and stimulated mononuclear cell culture supernatants were also higher than those of PMNL cultures with *E. faecalis* culture supernatants alone. In conclusion, increased PMNL functions by mononuclear cells may have possible contributions to tissue damage in the endodontic infections with *E. faecalis*. Additionally, soluble factors produced by active mononuclear cells may indirectly contribute to this process.

Key words: *Chemotaxis, Enterococcus faecalis, oxidative burst*

ÖZET

Enterococcus faecalis'in PMNL fonksiyonları üzerine etkisi: bir in vitro çalışma

Bu çalışmanın amacı, kuyucuklu sistem ve akım sitometri kullanarak *E. faecalis*'in polimorfonükleer lökositlerin (PMNL) kemotaktik ve oksidatif patlama aktiviteleri üzerine olan etkilerini değerlendirmekti. Bu mikroorganizmanın PMNL fonksiyonları üzerine etkilerini tespit etmek için, altı sağlıklı vericiden hazırlanan PMNL kültürleri *E. faecalis* kültür süpernatanı ilave edilerek veya ilave edilmeden inkübe edildi. İlave olarak, aktive mononükleer hücrelerden kaynaklanan çözünür faktörlerin *E. faecalis*'e karşı PMNL fonksiyonları üzerine etkileri kültür koşullarında test edildi. Sadece *E. faecalis* kültür süpernatanı ile hazırlanan veya beraberinde uyarılmış MNH kültür süpernatanları içeren PMNL kültürlerinde kemotaktik ve oksidatif patlama aktiviteleri, *E. faecalis* ve uyarılmış mononükleer hücre kültür süpernatanları içermeyen PMNL kültürlerinden daha yüksekti. *E. faecalis* kültür süpernatanı ve beraberinde uyarılmış MNH kültür süpernatanı içeren PMNL kültürlerinde oksidatif patlama aktivitesi, sadece *E. faecalis* kültür süpernatanı içeren PMNL kültürlerinden de yüksekti. Sonuç olarak, *E. faecalis* nedenli endodontik enfeksiyonlarda artmış PMNL fonksiyonları doku hasarına katkıda bulunabilir. Ayrıca, aktive mononükleer hücrelerden üretilen çözünür faktörler dolaylı olarak bu sürece katkı sağlayabilir.

Anahtar kelimeler: *Kemotaksis, Enterococcus faecalis, oksidatif patlama*

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Introduction

Bacteria are the main causative factor in the development of periapical inflammation (1,2). The main objective of endodontic therapy is, therefore, to eliminate bacteria from the infected root canal and to prevent reinfection. Complete chemomechanical preparation and careful obturation are essential to avoid reinfection of the root canal space. Even after cleaning, shaping and irrigation with disinfectants, total elimination of bacteria is difficult to achive complete debridement in all cases (3). However, these procedures undoubtly reduce the number of viable microorganisms in the root canal system. Therefore, reinfection or chronic periapical inflammation of root-filled teeth may be due to residual bacteria within the root canal system and dentinal tubules (4,5).

Various bacteria can be isolated within an infected root canal system where anaerobes predominate (6). These microorganisms are generally present in all parts of the root canal system, and found at varying depths within the dentinal tubules from the pulpal end (7,8). Berutti et al. have shown that bacteria is found within dentinal tubules in deep regions of dentine even after the root canal irrigation (8). Accordingly, in an *in vitro* study by Weigner et al. penetration time of bacteria into human root dentine up to a depth of at least 150 μm was found to be 4 weeks (9). There are regional variations in the extent of bacterial invasion of dentinal tubules in the infected root canals (10). In turn, cervical tubules are invaded to a greater extent than the midroot tubules, which are invaded more than those of the apical region.

E. faecalis is gram-positive, facultatively anaerobic, coccal bacterium that causes a wide variety of infections in humans (11). These enteric bacteria are also part of the normal flora of the oral cavity and can be present in the infected root canal during endodontic treatment (12). *E. faecalis* is the most common and occasionally the single isolated bacteria from root ca-

nals of teeth with persistent periapical periodontitis (13,14). Especially, these single species of organisms were found to be one of the predominant bacteria in the teeth in which root canal treatment failed (15). Pinheiro et al. have found *E. faecalis* in 52.94% of canals with bacterial growth (16). This microorganism has demonstrated the capacity to survive in an environment in which there are scant available nutrients and in which commensality with other bacteria is minimal (17). There have been many studies to identify the possible mechanism that would explain how *E. faecalis* could survive and grow within root canal system and reinfect an obturated root canal (18). It was shown that *E. faecalis* persists in harsh environmental conditions which exist in the endodontically treated tooth due to inherent antimicrobial resistance and adaptation to changed environment conditions (19).

Many kinds of immune cells such as polymorphonuclear neutrophils (PMNs), osteoclasts, lymphocytes, plasma cells, monocytes, macrophages, epithelial cells play a role in the state of early periradicular periodontitis via cell-to-cell contact or their secretory products (20). This pathological process is modulated by interactions between the cellular and soluble components of the immune systems. PMNs are the first cells that migrate into the tissues in response to invading pathogens. It is well known that a variety of neutrophil chemoattractants produced by different cells sources such as host endothelial, epithelial, and stromal cells are involved in PMNL migration to the site of infection. In this context, IL-8 is a soluble chemoattractant produced by endothelium in response to infectious agents and activates neutrophils (21). However, not only immune cells and their secretory products but also certain bacterial substances play a crucial role in the inflammation process. It has previously been reported that most bacteria produce a heterogeneous mixture of neutrophil chemotactic agents and contribute to neutrophil recruitment to the site of infection during inflammatory response (22). Sannomiya et al. have demonstrated that *E. faecalis* derived nonformylated peptides, in particular cAM373 and cPD1, are potent chemotactic factors and inducers of lysosomal granule enzyme secretion for rat peritoneal neutrophils (22).

Although there have been many investigation related to immunity against *E. faecalis,* immunopathogenic mechanisms of persistence of these microorganisms in root canals are still unknown. Therefore, we investigated in vitro effects of *E. faecalis* on PMNL functions in the present study. In addition, we studied whether phytohemagglutinin (PHA)-stimulated mononuclear cell (MNC) culture supernatants could affect neutrophil functions against *E. faecalis.*

Material and Methods

Donors: This study was carried out on 3 male and 3 female healthy donors, aged between 27 and 39 years. They had no immunological or metabolic disorders and took no medication that could alter the immunological parameters. All subjects were informed about the aim and procedures of the study and gave their consent.

Preparation of E. faecalis culture supernatants: E. faecalis strain ATCC29212 was cultured in brain heart infusion broth. Bacteria were grown at 37˚C in aerobic condition (85% N_2 and 5% CO_2) for 3 days. Bacterial cell suspensions were harvested and washed in 20 ml of phosphate-buffered saline (PBS) containing the protease inhibitor phenylmethylsulonyl-fluoride (PMSF, Sigma Chemical Co., St. Louis, MO, USA). Washed *E. faecalis* were disrupted by sonication (Sonic Dismembrator, Model 550, Fisher Scientific, Pittsburgh, PA, USA) on ice for 5 minutes with 30-sec pulses. The homogenate was centrifuged at 12.000 xg in a Sorvall RC5C (Sorvall İnstruments, DuPont, Wilmington, DE, USA) for 20 minutes at 4˚C. Then, the supernatant was collected, and stocked in deep freezer at -20˚C. Frozen samples were allowed to thaw at room temperature before use (23).

Preparation of human PMNL cultures: Peripheral blood samples from six healthy donors were drawn in sterile heparin-treated tubes. Immediately after phlebotomy, PMNLs and MNCs isolated from the whole blood samples through density gradient centrifugation. Double gradient of Ficoll-Hypaque Separating Solutions (histopaque 1077 and 1119, Sigma Chemical Co., St. Louis, MO, USA) were used for this purpose. Initially, histopaque 1077 (3 ml) was carefully layered onto the histopaque 1119 (3 ml). Then, 6 ml of whole blood was placed onto the upper gradient of tube and centrifuged at 700 g for 30 minutes at room temperature. PMNL and MNC layers were harvested from the interfaces by the aid of sterile Pasteur pipette and washed twice with RPMI-1640 medium (Gibco, Invitrogen Corporation, Scotland, UK), separately. They were then resuspended in 2 ml of same medium. The viability of both cell populations was more than 97% by staining with acridine-orange and ethidium bromide. These cell populations were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA). In this way, the percentages of PMNLs and MNCs in cell suspensions were found to be 99.6% and 98.8%, respectively. PMNL and MNC suspensions were adjusted to 1×10^6 cells/ml by RPMI,

and supplemented with 10% heat-inactivated newborn calf serum (NCS) (Gibco), 100 IU/ml penicillin, 100 mg/ml streptomycin (Sigma Chemical), and 2 mM/ml L-glutamin (Gibco) (19,23,24).

Firstly, stimulated and unstimulated MNC culture supernatants were prepared for using PMNL cultures. For this purpose, MNCs cultured in 24-well plates (Nunc Brand Products) in the absence or presence of phytohaemagglutinin-M (PHA-M, Gibco) at 37°C in a humidified 5% CO_2 atmosphere for 4 hours. PHA-M was used at a final concentration of 15 μg/ml in the cultures. At the end of 4 hours, the MNC cultures were centrifuged at 400 g for 10 minutes at room temperature. Then, culture supernatants from stimulated and unstimulated samples were separately pooled and aliquoted for PMNL cultures. The levels of interferon (IFN)-γ in culture supernatants were measured by enzyme immunoassay (DIAsource, Nivelles, Belgium).

In first two sets of experiments, PMNL cultures were incubated with RPMI or *E. faecalis* culture supernatants at 37ºC in a 5% CO_2 humidified atmosphere for 4 hours. The volumes of RPMI or *E. faecalis* culture supernatants were equivalent to those of PMNL cultures in these experiments. In third set of experiments, PHA-stimulated MNC culture supernatants of donors were added PMNL cultures with *E. faecalis* culture supernatants and the incubation was maintained for 4 hours at 37ºC in a 5% CO_2 humidified atmosphere. So, the effects of soluble factors derived from active MNCs on the PMNL functions against *E. faecalis* were tested. Culture supernatants and PMNL cultures were used in equal volume in third set of experiments. Optimal volumes determined in our previous experiences were used in these experiments (data not shown).

Functional analysis of neutrophils: The Transwell (TW) system consisting of inserts (containing 3 mm polycarbonate membrane) and 24-well plates were used for migration experiments. After the inserts were washed with RPMI into the wells of plates, they were transferred to other wells. Interleukin-8 (IL-8) (RD Systems, Minneapolis, MN, USA) was used as a chemoattractant and put in the wells in 500 μl of RPMI medium. The concentrations of IL-8 were adjusted according to the manufacturer's instructions. This chemoattractant was not put in control wells. First, 500 μL of cell suspensions ($1x10^6$ PMNLs/ml) obtained at sets of experiments were added into each TW insert and placed into the 24-well plate, which had already been filled with 500 μL of RPMI medium or chemokine. Then, TW apparatus were incubated in a 37°C humidified CO_2 incubator for 2 hours. After

2 hours of incubation, the TW inserts were lifted, and bases of the inserts were washed with RPMI medium. Migrated cells in the wells were counted with a hemocytometer. At the end of this process, the viability of the cells was more than 95% (21-24).

The evaluation of oxidative burst activity of PMNLs was performed by flow cytometry Bursttest kit (Orpegen Pharma, Heidelberg, Germany) used for quantitative determination of neutrophil oxidative burst. It contains unlabelled opsonized *E. coli* bacteria as particulate stimulus, phorbol 12-myristate 13-acetate (PMA) as high stimulus, and N-formyl-MetLeuPhe (fMLP) as low physiological stimulus, and dihydrorhodamine (DHR) as a fluorogenic substrat. PMNLs obtained at the end of the second set of experiments were incubated with the various stimuli at 37ºC, a sample without stimulus was used as negative control. Upon stimulation, the PMNLs that produced ROS were then analyzed as well as their mean fluorescence intensity (MFI) (21-24).

Statistical analysis: All statistical analyses were performed by using SPSS (SPSS 10.0) package. The Friedman test was used for multiple statistical comparisons. However, the Wilcoxon signed rank test was used to compare two paired samples. P values less than or equal to 0.05 were evaluated as statistically significant.

Results

Detectable IFN levels were only observed in two unstimulated MNC cultures (53 pg/ml and 104 pg/ml, respectively), whereas all stimulated MNC cultures had detectable IFNγ levels (mean±SD: 621±329 pg/ml; minimum-maximum: 123-989 pg/ml).

Chemotactic activity was found significantly higher in PMNL cultures with *E. faecalis* culture supernatants than those of PMNL cultures without *E. faecalis* and MNC culture supernatants (33±8% vs 26±9%, p<0.05) (Figure 1a). PMNL cultures with *E. faecalis* and stimulated MNC culture supernatants had also higher chemotactic activity compared to PMNL cultures without *E. faecalis* and stimulated MNC culture supernatants (40±5% vs 26±9%, p<0.05). There was no statistically significant difference between PMNL cultures with *E. faecalis* culture supernatants and with *E. faecalis* and stimulated MNC culture supernatants with respect to chemotactic activity (33±8% vs 40±5%, p>0.05).

Oxidative burst activity was found significantly higher in PMNL cultures with *E. faecalis* culture supernatants than those of PMNL cultures without *E. faecalis* and stimulated MNC culture supernatants (461±159 MFI vs 342±123 MFI, p<0.05) (Figure 1b).
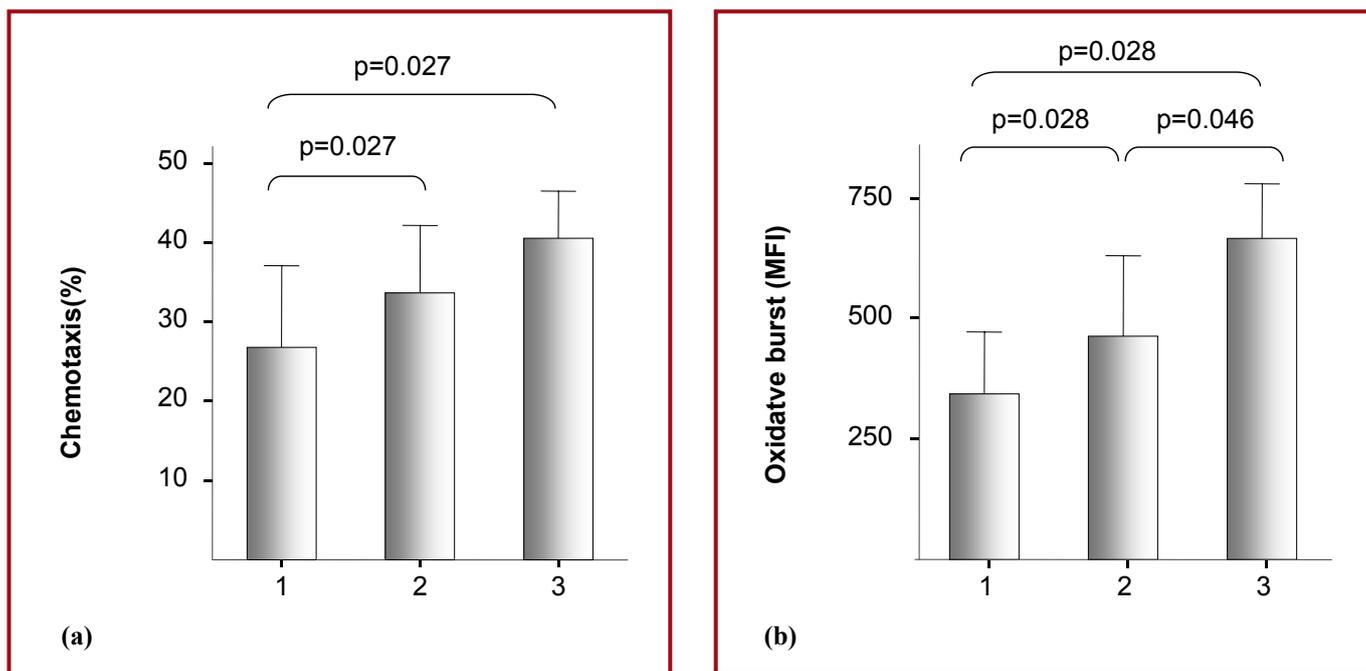
Figure 1. Chemotactic (a) and oxidative burst activity (b) of PMNLs. Bars in Mean±SD notation represent the data from unmixed PMNL cultures (1) and PMNL cultures mixed with E. faecalis culture supernatants alone (2) or combined with stimulated MNC culture supernatants (3). p values were indicated above the bars when there was a level of significance less than or equal to 0.05.

PMNL cultures with *E. faecalis* and stimulated MNC culture supernatants had also higher oxidative burst activity compared to PMNL cultures with *E. faecalis* culture supernatants and without *E. faecalis* and stimulated MNC culture supernatants (665±109 MFI vs 461±159 MFI and 665±109 MFI vs 342±123 MFI, respectively, $p < 0.05$).

Discussion

In the present study, we evaluated the direct effect of *E. faecalis* on the functions of PMNLs, using PMNL cultures. To assess the effect of *E. faecalis* on the priming of neutrophils, we used PMNLs incubated in an environment that was rich in cytokines secreted by stimulated MNCs. To attain such an environment, MLCs were stimulated with PHA, which has been known to induce the secretion of several cytokines, including IFNγ and TNFα.

Our results showed that *E. faecalis* derived substances increased chemotactic and oxidative burst activities of PMNLs in the cultures with or without MNC secretory products. These findings support the studies by Sannomiya et al. and Ember et al. who revealed that some of the *E. faecalis* derived substances, sex pheromones and their inhibitory peptides, were found to be chemotactic for human and rat neutrophils and also to induce superoxide production and lysosomal enzyme secretion (22,25). These peptides are one of the most cited virulence factors of *E. faecalis* related to endodontic infection and the periradicular inflammatory response (26). Aggregation substance, surface adhesins, lipoteichoic acid, extracellular superoxide production, the lytic enzymes gelatinase and hyaluronidase, and the toxin cytolysin are the other important virulance factors of *E. faecalis* (26). Superoxide anion derived from *E. faecalis* is also a highly reactive oxygen radical involved in cell, and tissue damage in inflammatory disease may react with a precursor in plasma to generate a factor that is chemotactic for neutrophils (27).

On the other hand, there are some reports in literature that seem contrary to our results. In the study of Shon et al., they have found that sonicated extracts of *E. faecalis* suppress PMNs recruiting activity by down-regulating α4 integrin expression, and proposed this microorganism may play a crucial role in persistent apical periodontitis (23,27). In addition, Vanek et al. have reported that *E. faecalis* aggregation substance promotes opsonin independent binding to human PMNLs via a complement receptor type 3 mediated mechanism (28). The resistance of this microorganism that gives damage human PMNLs has been explained with this non-opsonic binding of *E. faecalis* to PMNs (29). Furthermore, both extracellular superoxide production and phagosomal oxidant production of PMNLs against *E. faecalis* were found to be higher than those against the control strains lacking aggregation substance (29). Thus, oxidative burst activity of PMNLs may be a possible contribution to tissue damage in the endodontic infections with *E. faecalis*.

We also demonstrated that MNC secretory products increase the oxidative burst activity of PMNLs induced by *E. faecalis*. In our previous study, we observed that migration ability and reactive oxygen species production of PMNLs were increased by MNCs in mixed cultures (30). A plenty of cytokines have been shown to act as priming agents for PMNs, including IL-8 and TNFα (31). IFNγ also has been demonstrated to enhance, or prime, increased reactive oxygen species production in combination with a secondary stimulus (32). In this context, Berton et al. were the first to observe the priming effect of IFNγ on PMN oxidative burst (33). In their study, increased O_2 consumption and O_2^- production of PMNLs against either fMLP, concanavalin A, or lipopolysaccharide were observed in PMNLs pretreated with IFNγ (33). However, it was shown that the priming effect of IFNγ on PMNLs may be specific to stimuli that act via membrane associated receptors. The other modulatory effect of IFNγ on PMNLs is to stimulate the production of the cytotoxic agent nitric oxide by a variety of cells, including macrophages and neutrophils, and to cause undesirable cell and tissue damage (34).

This in vitro study supports the previous studies on the effects of *E. faecalis* on neutrophil functions. In view of our findings, we suggest that a direct effect of *E. faecalis* on neutrophil functions seems improbable. MNC products such as IFNγ play an important role in innate immune response by priming neutrophils that lead to an enhanced antimicrobial activity. As a consequence of these results, increased PMNL functions by *E. faecalis* and MNC products may have possible contributions to tissue damage in the endodontic infections with *E. faecalis*. However, in the future, other comparative *in vivo* and *in vitro* studies including patients and healthy individuals should be carried out to explain different clinical courses of the endodontic infections by *E. faecalis*.

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