Effect of Sonicated Extracts of *Enterococcus faecalis* on the Production of Matrix Metalloproteinase-8 by Human Polymorphonuclear Neutrophils

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ABSTRACT

This in vitro study monitored MMP-8 production on PMN by stimulated with the following three groups; Sonicated extracts of *E. faecalis* (SEF), SEF treated with $Ca(OH)_2$ (12.5㎎/㎖) for 7 days, and lipopolysaccharides (LPS) of *E. coli*. The level of MMP-8 in each group was immediately measured by ELISA. The data were analyzed with Kruskal-Wallis test and Mann-Whitney U test.

In the SEF group, the level of production of MMP-8 was higher than the negative control group in low concentration (0.05㎍/㎖) of SEF ($p < 0.05$), but it decreased with an increase in the concentration of SEF ($p < 0.05$). In the case of SEF treated with $Ca(OH)_2$, all of the MMP levels were higher than negative control group ($p < 0.05$), but no statistical difference was found among the different SEF concentrations ($p > 0.05$). All of the levels in *E. coli* LPS were increased with increasing concentrations ($p < 0.05$).

According to this study we could summarize as follows:

1. MMP-8 was expressed at low level in untreated PMN group and the levels of MMP-8 were upregulated in PMN stimulated by *E. coli* LPS groups.
2. In the SEF groups, the level of production of MMP-8 decreased with an increase in the concentration of SEF ($p < 0.05$). So *E. faecalis* may have suppressive effect on the production of MMP-8 by PMN.
3. In the case of SEF treated with $Ca(OH)_2$, all of the MMP levels at different SEF concentrations were higher than untreated PMN group ($p < 0.05$), but no statistical difference was found among the different SEF concentrations ($p > 0.05$). 〔J Kor Acad Cons Dent 30(2):138-144, 2005〕

Key words : *E. faecalis*, MMP-8, Calcium hydroxide, PMN leukocyte

- received 2005. 1. 21, revised 2005. 3. 6, accepted 2005. 3. 7 -

Ⅰ. INTRODUCTION

Bacteria and their by-products are considered to be the primary etiologic agents of pulpal necrosis and periapical lesions[1]. Therefore, endodontic treatment aims at eliminating of microorganisms from the root canal system and preventing of reinfection. However some cases fail even when apparently well treated. In fact, a number of factors associated with failure of endodontic therapy. But, most treatment failures are caused by microorganisms persisting in the apical parts of root canals of obturated teeth. It seems to be bacterial effects in root canal and periapical lesion.

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Especially, single spices of gram-positive organisms, *Enterococcus faecalis* had been found to be one of predominant bacteria in teeth in which root canal therapy failed[2].

So, the use of intracanal medication to disinfect the root canal system has been advocated[3]. Calcium hydroxide ($Ca(OH)_2$) has been the intracanal medication of choice. Sjögren et al.[4] and byström et al.[3] reported that 7-days and 30-days applications of $Ca(OH)_2$, respectively, eliminated bacteria that survived instrumentation in root canals. However, in case of *E. faecalis*, $Ca(OH)_2$ dressing is ineffective[3]. Therefore it seems that the frequent isolation of Enterococci in failed endodontic cases puts in question the routine use of $Ca(OH)_2$ as an intracanal medication[5]. Survival of *E. faecalis* in $Ca(OH)_2$ at high pH was related to a functional proton pump[7]. However few researches reported how these factors affect the antibacterial activity of $Ca(OH)_2$.

Matrix metalloproteinases (MMPs) form a family of structurally related but genetically distinct endopeptidases expressed at low level in normal tissue, but upregulated during inflammation[8]. They are group of zinc-dependent proteinases, secreted or released by host cells (ie, PMN, macrophage, bone cell, fibroblast) as proenzymes. There are multiple members of family, including interstitial collagenase (MMP-1), neutrophil collagenage (MMP-8), stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), gelatinase B (MMP-9). Shin et al.[9] reported that MMPs play an important role in the pulp tissue destruction in inflamed pulp and periapical lesion. MMP-8 of them has been thought to be uniquely produced by developing PMN cells in bone marrow, and that the MMP-8 activity is dependent on the release of the enzyme from PMN cells by degranulation[10]. However, recent findings demonstrated expression of MMP-8 in mesenchyme derived, non-PMN lineage cells, including dental pulp fibroblasts, odontoblasts, inflamed epithelial cells and plasma cells[11-12]. In other research, MMP-8 is reported that they have an important role in the degenerations of the extracellular matrix in inflamed pulps and periapical lesions[13].

In this study, the purpose was to examine the levels of production of MMP-8 by PMN stimulated with Enterococcus faecalis and effect of $Ca(OH)_2$ on their production.

Ⅱ. MATERIALS AND METHOD

Preparation of bacterial sonicated extracts

Enterococcus faecalis (ATCC 29212) strains were used in this study. Strains were grown in 1-liter cultures in 85% N2-10% H2-5% CO_2 chamber for 3 days at 37℃. The medium used was 3.7% brain heart infusion broth supplemented with 0.5% yeast extract, 0.5mg L-cysteine hydrochloride, and 0.5% sodium bicarbonate.

Bacterial fractions were prepared. Briefly, bacterial cell harvest from 1-liter cultures were washed and suspended in 20ml of phosphate-buffered saline (PBS). To minimize the loss of protein, 1mK of Phenylmethylsulfonylfluoride (PMSF) were added. PMSF act as protease inhibitor at protein extraction. And suspensions of bacterial cell were disrupted by sonication (100W output, Fisher sonicator) on ice for 5min with 30-sec pulses on and 10-sec pulse-off in the presence of glass beads. Sonicated debris was centrifuged at 12,000 × g in a Sorvall RC5C (Sorvall Instruments, DuPont) for 20 min, and membrane fraction was sedimented by ultracentrifugation at 85,000 × g for 60 min at 4℃. To investigate protein profile of sonicated extracts of *E. faecalis* (SEF), supernatant was filtered with 0.22㎛ syringe and separated by 10% SDS-PAGE at 100V for 90min, and the protein concentration was determined by Bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). The SDS-PAGE reveals that the soluble cytoplasmic protein of *E. faecalis* strain ATCC 29212 is mainly consist of 75kDa and 35-50kDa polypeptide. The pellet was resuspended in 20㎖ of 10mM Tris buffer (pH 7.4), and stocked in deep-freezer at -20℃.

Sonic extract of *E. faecalis* (SEF) treated with $Ca(OH)_2$

Each of SEF was suspended to 100㎍/㎖ with pyrogen-free water. $Ca(OH)_2$ powder (Sigma Chemical Co., St Louis, MO, USA) 12.5㎎/㎖ was added. All samples were vortexed for 20 seconds once a day and incubated at 37℃ for 7days. After that, upper part of

solution was ultrafilterated (Centriprep YM-10, 10,000 NMWL, Millipore Co., USA) and stocked in deep-freezer at -20℃.

Preparation of PMN

Using heparinized tubes, Venous blood was obtained from 6 healthy volunteers (26~35years). The erythrocytes of the obtained blood were segregated through 6% dextran (Mwt. 500,000, Sigma Chemical Co., St Louis, MO, USA), the leukocytes were extracted after centrifugation using lymphoprep (Nycomed Pharma AS, Oslo, Norway) and remained erythrocytes were eliminated by carrying out hypotonic lysis. After verification through the Trypan blue dye exclusion test & Giemsa staining, the PMN leukocytes that were obtained by this method proved to have 98% or more vitality and 97% or more purity. One millimeter of the extracted PMN leukocytes were 24 well plate with each well having a cell account of 10×10^4.

Bacterial stimulation and MMP-8 production

SEF group and SEF treated with $Ca(OH)_2$ group and *E.coli* LPS were diluted at three concentration. And PMN media were stimulated by each groups and control group was not activated. Final concentrations of each group were 0.05㎍/㎖, 0.5㎍/㎖ and 5㎍/㎖ and incubated at 37℃ for 30 minutes.

Enzyme-linked immunosorbent assay (ELISA)

After incubation, the amount of MMP-8 present in the culture supernatants were assayed by Enzyme-linked immunosorbent assay (ELISA, R and D System, Minneapolis, USA) according to the manufacturer's protocols.

Statistical analysis

Data were statistically analyzed using the Man-Whitney rank sum test and Kruskal-wallis test. A value of $P < 0.05$ was considered statistically significant.

Ⅲ. RESULTS

The results of this study are the mean values of six samples of duplicate cultures and summarized in Table 1, Figure 1 and Figure 2.

In the SEF groups, the level of production of MMP-8 was higher in comparison to the negative control group in low concentration (0.05㎍/㎖) of SEF ($P < 0.05$), but it decreased with an increase in the concentration of SEF ($P < 0.05$). In the case of SEF treated with $Ca(OH)_2$, all of the MMP levels at different SEF concentrations were higher than control group ($P < 0.05$), but no statistical difference was found among the different SEF concentrations ($P > 0.05$). All of the levels in *E. coli* LPS were increased with increasing concentrations ($P < 0.05$).

Table 1. Average concentration(±SD) of MMP-8

Group	Mean concentration (±SD, ㎍/㎖ protein)		
	0.05㎍/㎖	0.5㎍/㎖	5㎍/㎖
SEF	2.3209 ± 0.0398*	2.1303 ± 0.1216	1.9447 ± 0.1310*
SEF treated with $Ca(OH)_2$	2.8775 ± 0.2100	2.9003 ± 0.0896	2.7745 ± 0.0851
E.coli LPS	2.4390 ± 0.0866+	2.6975 ± 0.1650+	2.7929 ± 0.2193
Untreated PMN		2.2497 ± 0.0041	

SEF = sonicated extract of *Enterococcus faecalis*

+ and * are statically significantly different ($P < 0.05$) between groups

SEF groups and SEF treated with $Ca(OH)_2$ groups are statically significant different ($P < 0.05$) in comparison with untreated PMN group.

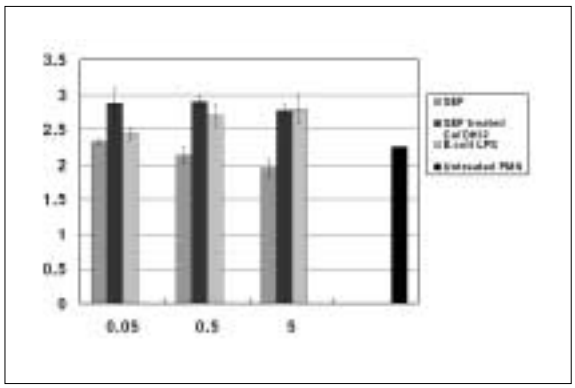

Figure 1. Average concentration of MMP-8 (± SD) (ng/mol)

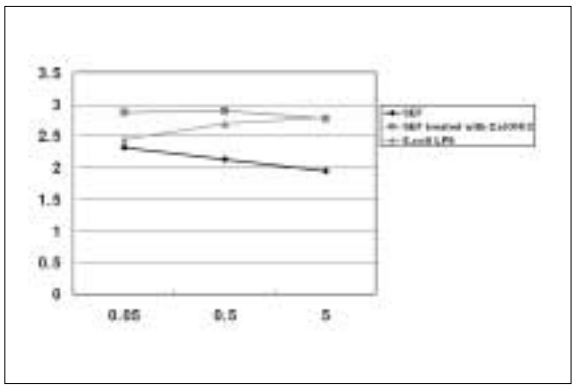

Figure 2. Average concentration of MMP-8 (ng/mol)

Ⅳ. DISCUSSION

In recent years, MMPs have gained considerable attention in many studies. MMPs play an important role in the pulp tissue destruction of acute inflamed pulp[9]. The role of MMP-8 synthesized by the cells in the pulp-dentinal complex is not clear. But MMP-8 was reported that they have an important role in the degenerations of the extracellular matrix in inflamed pulps and periapical lesions[13]. Therefore, a hypothesis was proposed that MMP-8 could be present in the periapical lesion, and the enzyme level could be related to the activity of the periapical tissue. And MMPs are endopeptidases expressed at low level in normal tissues, but upregulated during inflammation[8]. So, untreated PMN had some MMP-8 in this study.

In previous studies, sonic extracts from bacteria were studied for their effects on the immune response. For example, sonicated material of *Fusobacterium nucleatum* can evoke a concentration-dependent stimulatory or suppressive effect on the proliferation rate of accessory cell[14]. Sonicated extract of *Actinobacillus actinomycetemcomitans* suppressed interleukin-2 production of T-cells[15]. Kim et al.[16] showed that sonicated extracts of *Enterococcus faecalis* also suppressed the production of interleukin-2 and interleukin-4 in lymphocytes. So in this study, SBE may have depressive effect to PMN. In positive control group, all of the MMP-8 levels in *E. coli* LPS were increased with increasing concentrations ($P < 0.05$).

In this study, MMP-8 production level at low level of SEF (0.05㎍/㎖) was seen a little elevation compare to untreated control group ($P < 0.05$). But it decreased with an increase in the concentration of SEF ($P < 0.05$). This phenomenon showed that ability of *E. faecalis* to impair the production of MMP-8 may be related to the suppression of PMN. This fact may be seen another immunosuppressive effect of SEF.

However, there is no study how *E. faecalis* have immunosuppressive effect exactly. But *E. faecalis* is the most prevalent species of enterococci in root canals of failed case[17] and for it to be involved in the pathogenesis and maintenance of apical periodontitis, it must survive in the root filled canals as where the nutrient supply is limited. *E. faecalis* has been shown to have relatively uncommon capacity to survive in root canals as a monoculture without the support of other bacteria[18]. *E. faecalis* is also known to survive periods of starvation in water[19] and infected dentin in water for periods of at least 10 days[20].

$Ca(OH)_2$ has been widely used as an intracanal medication to eliminate bacteria and is currently acknowledged to be one of the most effective antimicrobial dressings used during endodontic therapy[21]. Although the exact mechanism of action of $Ca(OH)_2$ is still unknown, its antimicrobial activity is generally considered to be related to the release of hydroxyl

ions in an aqueous environment, producing a pH of approximately 12.5, even in very dilute mixture. Most endodontic pathogens are unable to survive in this alkaline environment[21]. The mechanism that $Ca(OH)_2$ uses to eliminate bacteria may include damage to bacteria cytoplasmic membrane by including lipid peroxidation, protein denaturation, and damage to bacterial DNA and by serving as physical barrier that withholds nutrients for bacterial growth and limits space for bacterial multiplication[22].

But *E. faecalis* is known to withstand a high pH. This is an identifying characteristic of *E. faecalis*. At pH 11.4 or greater, *E faecalis* does not survive, yet it can survive at a pH below 11.5 as shown in this and earlier studies[3]. Because of the buffering effect of dentin[23-25], it is unlikely that the high pH of $Ca(OH)_2$ (〉 11.5) is attained within dentinal tubules where *E. faecalis* has the capacity, at least in vitro, to penetrate deeply[20,26-28]. In radicular dentin, alkalinity may only reach pH 10.3 after dressing the canal with $Ca(OH)_2$ [24]. An adaptive response in alkaline pH and stress-induced protein synthesis appear to play minor roles in cell survival, a functional proton pump with the capacity to acidify the cytoplasm is critical for survival of *E. faecalis* at high pH[7].

So considered *E. faecalis* in endodontic treatment, additive medication is recommended. For example, chlorhexidine (CHX) is a broad-spectrum antimicrobial agent that has been reported to be an effective medicament in endodontic treatment therapy[29]. As a root canal irrigant and intracanal medicament, CHX has an antibacterial efficacy comparable to that of sodium hypochlorite (NaOCl)[30-31]. In addition, it is also effective against strains resistant to $Ca(OH)_2$[32]. Therefore the use of combination of $Ca(OH)_2$ and CHX is recommended in endodontic treatment.

In our study, $Ca(OH)_2$ had some effect to production of MMP-8 by PMN. But comparison to untreated PMN group, this data does not support that $Ca(OH)_2$ removed immunosuppressive effect of SEF. MMP-8 levels of $Ca(OH)_2$ treated SEF group had another impression, this may because of the high pH of $Ca(OH)_2$. Gram positive microorganisms have external membrane structure, cell wall. This cell wall have two important high molecule, peptidoglycan and teicoic acid. In this study, SEF treated with $Ca(OH)_2$ for 1 week showed different appearance in the production of MMP-8. This result means that $Ca(OH)_2$ could possibly modified SEF. The high pH of $Ca(OH)_2$ may modify acidity of teicoic acid. But no statistical difference was found among the different concentrations ($P > 0.05$).

In our study, this result suggested that bacterial protein sonicated extract of *E. faecalis* can inhibit the immune response by PMN and this could be one of possible factors why *E. faecalis* are found frequently in the teeth with failed endodontic treatment. The use of an antimicrobial agent as intracanal medication between appointments may increase the chances for successful endodontic treatment by reducing residual bacteria in root canal system. In endodontic treatment, $Ca(OH)_2$ may have limited antibacterial effect to *E. faecalis*. So our study suggests that the use of only $Ca(OH)_2$ in clinical endodontic treatment may not be effective to *E. faecalis*.

Ⅴ. CONCLUSION

According to this study we could summarize as follows:

1. MMP-8 was expressed at low level in untreated PMN group and the levels of MMP-8 were upregulated in PMN stimulated by *E. coli* LPS groups.
2. In the SEF groups, the level of production of MMP-8 decreased with an increase in the concentration of SEF ($p < 0.05$). So *E. faecalis* may have suppressive effect on the production of MMP-8 by PMN.
3. In the case of SEF treated with $Ca(OH)_2$, all of the MMP levels at different SEF concentrations were higher than SEF & untreated PMN groups ($p < 0.05$), but no statistical difference was found among the different SEF concentrations ($p > 0.05$).

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국문초록

ENTEROCOCCUS FAECALIS 추출물이 다형핵 백혈구의 METALLOPROTEINASE-8 분비에 미치는 영향에 관한 연구

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*Enterococcus faecalis*의 추출물을 성인의 말초혈액으로부터 얻은 인간의 백혈구를 이용해 Matrix Metalloproteinase-8 (MMP-8)의 생산에 미치는 영향에 대해서 알아보았다.

*Enterococcus faecalis*를 배양한 뒤 초음파 분쇄를 하여 추출물을 얻어냈다. 이중 일부는 적정한 농도의 $Ca(OH)_2$를 처리하였다. 이후 *E. faecalis* 추출물군, *E. faecalis* 추출물에 $Ca(OH)_2$ 를 처리한 군으로 분리하여 다형핵백혈구를 자극하여 MMP-8의 생산량을 측정하였다.

MMP-8는 처리하지 않은 PMN group에서도 나타났고, PMN을 *E. coli* LPS로 자극시킨 경우 증가하였다 ($p < 0.05$). SEF group 들에서는 MMP-8의 생성량이 SEF 의 농도가 증가함에 따라 감소하였다 ($p < 0.05$). 따라서 *Enterococcus faecalis*는 PMN의 MMP-8 생산 억제 효과를 가진다고 볼 수 있다. $Ca(OH)_2$로 처리한 SEF 군의 경우 각기 다른 SEF 농도에서도 모두 MMP-8 생성량이 아무 처리도 하지 않은 PMN 군보다 많았다 ($p < 0.05$). 하지만 각 군들 사이에 통계적 차이는 없었다 ($p > 0.05$).

주요어 : *E. faecalis*, MMP-8, 수산화칼슘, 다형핵백혈구