Research Article

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Effects of calcium hydroxide and N-acetylcysteine on MMP-2, MMP-9, TIMP-1 and TIMP-2 in LPS-stimulated macrophage cell lines

Kalsiyum hidroksit ve N-asetilsistein’ in LPS ile stimüle edilmiş makrofaj hücre hatlarındaki MMP-2, MMP-9, TIMP-1 ve TIMP-2 üzerine etkileri

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Abstract

Aim: This study was evaluated the effects of N-acetyl-cysteine (NAC) and calcium hydroxide (Ca(OH)₂) on the expression levels of matrix metalloproteinase -2, -9 (MMP-2, -9) and tissue inhibitor metalloproteinase -1, -2 (TIMP-1, -2) in lipopolysaccharide (LPS)-stimulated human macrophages.

Methods: Human monocyte precursor cells (THP-1) were differentiated into macrophage-adherent cells and were stimulated with LPS for 24 h. Then individually incubated with NAC or Ca(OH)₂ for 24, 48 and 72 h. Following incubation, protein expression and mRNA levels of MMP-2, -9 and TIMP-1, -2 were evaluated using enzyme-linked immunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction (qRT-PCR). Data were statistically analysed using two-way ANOVA, to followed by Bonferroni test at α = 0.05.

Results: NAC significantly decreased mRNA expression and protein levels of MMP-9, while Ca(OH)₂ decreased mRNA expression alone at 24 h. NAC and Ca(OH)₂ decreased mRNA expression of MMP-2 at 24 h, while NAC increased this expression at 48 h. Although NAC and Ca(OH)₂ decreased the mRNA expression of TIMP-1, -2 at 24 h, only NAC increased mRNA expression of TIMP-1 at 48 h.

Conclusion: At the early stages of inflammation, NAC and Ca(OH)₂ have anti-inflammatory effects on macrophages.

Keywords: NAC; Ca(OH)₂; MMP-2; MMP-9; TIMP-1; TIMP-2; Macrophage cell line.

Özet

Amaç: Bu çalışmanın amacı lipopolisakkaritle (LPS) stimule edilmiş insan makrofaj hücrelerinde N-asetilsistein (NAC) ve kalsiyum hidroksit’in (Ca(OH)₂), matriks metalloproteinaz-2 ve -9 (MMP-2, -9) ve doku inhibitor metalloproteinaz-1 ve -2 (TIMP-1, -2) salınımı üzerine etkilerini belirlemektir.

Yöntemler: İnsan monosit precursor hücreleri (THP-1) makrofaj adherent hücrelere dönüştürülmüştür. Makrofaj hücreleri 24 saat LPS ile stimüle edilmiş NAC veya Ca(OH)₂ ile 24, 48 ve 72 saat inküбе edilmişlerdir. İnkübasyonu takiben, MMP-2, -9 ve TIMP-1, -2 mRNA seviyeleri ve protein salınımını enzim ilintili immün test (ELISA) ve kantiyatif eş zamanlı polimeraz zincir reaksiyonu (qRT-PCR) ile değerlendirilmiştir. Veriler istatistiksel olarak Bonferronni testini takiben (α = 0.05) iki yönlü varyans analizi (ANOVA) ile analiz edilmiştir.
Introduction

Bacterial infection within the root canal induces tissue breakdown around the apical area as a result of local immune response by degradation of several extracellular matrix (ECM) components such as collagen, fibronectin and laminin [1–3]. ECM components mediate tissue remodeling as well as cell growth and differentiation and are destroyed by matrix-degrading enzymes called matrix metalloproteinases (MMPs). MMPs represent a family of Zn$^{++}$ and Ca$^{++}$ dependent endopeptidases, including collagenases, gelatinases and stromelysins [4–6]. MMP-2 and MMP-9, also called gelatinases, play crucial roles in degradation of collagen and other ECM components [3, 4]. During tissue remodeling, MMP activity is primarily regulated by tissue inhibitor metalloproteinases (TIMPs), which may inhibit active forms of all MMPs. Four different TIMPs have been identified. The primary inhibitors of MMP-9 and MMP-2 are TIMP-1 and TIMP-2 [5, 6].

Studies have reported that gelatinases play a vital role in pulp and periradicular tissue breakdown that occurs during the initial phase of lesion development [7–12]. During root canal treatment of teeth with periradicular lesions, calcium hydroxide (Ca(OH)$_2$) is primarily recommended as an intracanal medicament in order to eliminate or reduce the bacterial contamination present in the root canal, and induce healing of periradicular tissues [13–15]. It was reported that Ca(OH)$_2$ significantly decreased mRNA expression of MMP-1 and increased that of TIMP-1, in addition to presentation of more organized ECM, when used as a root canal dressing [14]. However, Ca(OH)$_2$ application has some disadvantages such as decreasing of dentin strength [16] and disruption of the adhesion between endodontic sealer and dentin [17]. Moreover, it was found to be cytotoxic and less effective against certain endodontic pathogens such as Enterococcus faecalis (E. faecalis) and Candida albicans [18–20].

In LPS-activated cells, inflammation and oxidative stress occur at the same time; antioxidants may inhibit these processes. N-acetylcysteine (NAC) is a thiol-containing compound that can act both as a precursor of reduced glutathione (GSH) and as a direct scavenger of reactive oxygen species (ROS). NAC is an important cellular antioxidant [21, 22]. In previous studies, NAC suppressed LPS-induced inflammatory responses in gingival fibroblasts [23] and macrophage cell lines [24] as well as prevention of alveolar bone loss in the rat model [25]. In addition, studies have reported to decrease the gelatinolytic activity in macrophage cell lines [26, 27]. NAC also has antibacterial effects against certain microorganisms and was found to be effective in both planktonic and biofilm forms of E. faecalis which is the major microorganism of failed root canal treatment [28].

In our previous publication, we have reported the pro- and anti-inflammatory effects of NAC and Ca(OH)$_2$, on lipopolysaccharide (LPS)-stimulated human macrophage cell lines and showed that NAC has antiinflammatory effect [24].

Therefore, we aimed to evaluate the effects of NAC, compared with Ca(OH)$_2$, using similar conditions with our previous study, on MMP-2, MMP-9 and their tissue inhibitors, TIMP-1 and TIMP-2, mRNA expression and protein levels in LPS-stimulated human macrophage cell lines.

Materials and methods

Preparation of compounds

After sterilization of Ca(OH)$_2$ (Sultan Healthcare, Hackensack, USA) and NAC (Sigma Chemical Company, St. Louis, MO, USA) powder under ultraviolet (UV), stock solutions of NAC and Ca(OH)$_2$ were prepared by dissolving these in RPMI-1640 medium (Roswell Park Memorial Institute) and stored at +4°C. The optimum concentrations of NAC and Ca(OH)$_2$ were determined according to our previous study by using flow cytometry analysis with propidium iodide (PI) regarding cell cytotoxicity that represents the viability of at least 50% cells [24].

Cell culture and treatment

In this study, human promonocytic cell lines (THP-1) (ATCC, Rockville, MD, USA) were cultured at 37°C in a
humidified incubator with 5% CO in RPMI-1640 medium with 10% foetal bovine serum (FBS), 1% penicillin, streptomycin and glutamine and plated in six-well culture plates at a density of $1 \times 10^6$ cells/mL. Subsequently, THP-1 monocyte cell lines were first incubated with phorbol myristate acetate (PMA) at a concentration of 400 nM for 72 h, followed by fresh medium without PMA for 96 h. Differentiated macrophage cells adhered to the flask, whereas undifferentiated monocytic cells in suspension were removed by washing with PBS (pH 7.4) [24].

For treatment, in order to completely prevent cells from apoptotic DNA fragmentation, the cells were incubated with culture media containing 10 mM NAC (NAC group) and 40 μg/mL Ca(OH)₂ (Ca(OH)₂ group) for 24 h. The control group did not receive any treatment. Following washing with PBS (1X) three times, the cells were incubated with 10 ng/mL Escherichia coli (E. coli) (Sigma Chemical Company, St. Louis, MO, USA) LPS, for 24, 48 or 72 h.

**Enzyme-linked immunosorbent assay (ELISA)**

Following LPS stimulation, the protein levels of MMP-9, TIMP-1, MMP-2 and TIMP-2 in the supernatant of macrophage cell lines were analysed using an ELISA reader (SpectraMax M2, Molecular Device, USA) with an ELISA kit (R&D Systems, USA) according to the manufacturer’s instructions at 450 nm and normalized with the standard solution. All experiments were performed in triplicate for two independent experiment sets.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

mRNA expression levels of MMP-9, TIMP-1, MMP-2 and TIMP-2 was determined using quantitative real-time polymerase chain reaction (qRT-PCR) assay. The RNeasy Mini kit (Qiagen, Hilden, Germany) was used for total RNA extraction according to the manufacturer’s instructions. RNA yield and quality were detected on the basis of spectrophotometric measurements at wavelengths of 260 and 280 nm with NanoDrop ND 1000 (Thermo Scientific, Wilmington, USA). Reverse transcription was performed using a Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics GmbH, Germany) with 1 ug total RNA according to manufacturer’s instructions. After cDNA synthesis was completed, samples were stored at −20°C. qRT-PCR analyses were performed in triplicates using the LightCycler 480 Probes Master kit on Light Cycler 480 II (Roche Applied Science). Amplification conditions of PCR cycles were 95°C for 10 s, 60°C for 30 s and 72°C for 1 s. The relative amount of the target gene was normalized relative to the control gene.

The following primer sequences were used in the RT-PCR reactions: hMMP-9 F: 5′CCTCTGGAGGTTGAGCTTG3′, hMMP-9 R: 5′CCTGGCAAAATAGGCTTTG3′; hTIMP-1 F: 5′CTGTGTGCTGCTGTGGCTGAT3′, hTIMP-1 F: 5′AACCTGGCCTGATGAGC3′; hMMP-2 F: 5′TATTTGATGGCATCGCTCAGG3′, hMMP-2 F: 5′CCAAATGGAACCGTCTTTG3′; hTIMP-2 F: hTIMP-2 R: hTIMP-2 R: 5′CCATCCAGGCACTCGT3′ and Hprt-1 Real-Time Ready single assay (Roche Diagnostics GmbH, Germany).

**Statistical analysis**

The results of the experimental groups were statistically analysed using two-way ANOVA followed by Bonferroni test for pair-wise comparisons with a significance level of $p < 0.05$.

**Results**

Two-way ANOVA revealed significant differences among the experimental groups ($p < 0.001$).

**MMP-9 assays**

Compared with the control group, NAC significantly decreased both mRNA expression and protein levels at 24 h, whereas Ca(OH)₂ only mRNA expression alone at 24 h ($p < 0.05$). However, at 48 h, NAC and Ca(OH)₂ increased mRNA expression as well as protein levels ($p < 0.05$; Figure 1A and B).

**TIMP-1 assays**

NAC and Ca(OH)₂ significantly decreased mRNA expression at 24 h compared with the control group ($p < 0.05$). In contrast, NAC increased the mRNA expression at 48 h compared with the Ca(OH)₂ and control groups ($p < 0.05$; Figure 1D).

**MMP-2 assays**

Compared with the control group, NAC and Ca(OH)₂ significantly decreased mRNA expression at 24 h ($p < 0.05$).
Figure 1: The time (24, 48 and 72 h) and medication NAC (10 mM) and Ca(OH)$_2$ (40 μg/mL) dependent protein and the mRNA expression levels of MMP-9, TIMP-1, MMP-2 and TIMP-2 on THP-1 macrophage cell line were shown in graphics. Values and error bars of average protein and mRNA expression levels represent the means and SD of experimental groups. The asterisks show the statically difference between groups by the Bonferroni test for pair-wise comparison at $\alpha = 0.05$. $1 \times 10^6$ cell were used in the experiment sets.
On the other hand, only NAC decreased mRNA expression compared with the control and Ca(OH)$_2$ groups at 72 h ($p < 0.05$). However, NAC increased the mRNA expression at 48 h compared with the control group ($p < 0.05$; Figure 1). 

**TIMP-2 assays**

NAC and Ca(OH)$_2$ significantly decreased mRNA expression at 24 h compared with the control group ($p < 0.05$). Moreover, Ca(OH)$_2$ decreased mRNA expression compared with the control and NAC groups at 72 h ($p < 0.05$; Figure 1). 

**Discussion**

In the present study, effects of NAC on gelatinolytic activity were evaluated in macrophage cell lines by comparing to Ca(OH)$_2$. NAC significantly decreased mRNA expression and protein levels of MMP-9. Moreover, during the same time intervals, NAC decreased only mRNA expression of MMP-2. However, NAC has been reported to decrease protein levels of MMP-2 in gingival fibroblasts [23] and endothelial cells [27]. The findings of the present study suggest that MMP-2 could not be translated to its protein owing to other facts affecting the signaling pathway, which might be depending on using different cell types and experimental procedures. According to a previous study, MMP-2 is primarily produced in vitro by fibroblasts and endothelial cells while MMP-9 by macrophages and other inflammatory cells [6]. Consistent with this information, the present study, MMP-9 and TIMP-1 expressions were high but MMP-2 expression levels are low in THP-1 macrophages.

In our previous publication, we have reported the effects of NAC and Ca(OH)$_2$ on TNF-α and TGF-β in LPS-stimulated macrophages cell lines. The results showed that NAC and Ca(OH)$_2$ significantly inhibited TNF-α expression at both the protein and mRNA expression level at the 4th h. It seems that both materials have a strong anti-inflammatory effect at the early phase of inflammation. These materials only increased TGF-β1, one of the cytokines involved in the wound repair process in periapical lesions, mRNA level at the 24th h [24].

Cytokines induce MMP release for matrix degradation and turnover [3, 6], TNF-α induces MMP-9 and MMP-2 expression in a variety of cell types including macrophages [29, 30]. Under the lights of our previous publication and literature results; we hypothesized that NAC could affect the gelatinolytic activity, which plays an important role in degradation of collagen and other ECM components during inflammation after cytokine release [3, 4, 15].

NAC is a dithiol that induces intracellular GSH synthesis and directly neutralizes ROS by donating a hydrogen atom from its thiol group [6, 21–23, 27] It was reported that NAC affect the MMP binding to the Zn atom to block catalysis [3, 4, 6]. Previous studies have indicated the possible role of NAC in the signaling pathway. In fact, ROS production leads to activation of ERK1/2 and subsequent activation of nuclear factor-kB (NF-kB). The promoter of MMP-9, acting as regulatory elements of transcription factors, has an NF-kB binding site inside, and NAC inhibits NF-kB activation directly or the promoter of MMP-9, which, in turn, blocks MMP-9 activity. On the other hand, NAC probably has a direct inhibitory effect on the gelatinolytic capacity of MMP-2 [27, 30].

In this experiment, NAC (10 mM) and Ca(OH)$_2$ (40 μg/mL) concentrations were selected according to our previous research that yielded 50% viable cells and compatible with literature results [26, 27]. It was reported that lower NAC concentrations had greater remodeling effect, whereas higher concentrations was expected to have better antibacterial effects [21]. NAC has been shown to effectively reduce biofilm formation in various gram-positive and gram-negative bacteria, especially for *E. faecalis* which is the major microorganism of persistant endodontic infections [28]. NAC was most bactericidal at pH 11 when combined with 2% chlorhexidine; [31]. Since pH adjustment might alter the real activity of NAC molecule, in present study, NAC was prepared at pH 7.4 to evaluate cellular MMP expressions for prolonged time periods.

In literature, the gelatinolytic activity of NAC has been evaluated mostly for 24 h [26, 27]; the results of these studies were consistent with the 24-h results of our study. On the other hand, in our study, NAC was not effective in inhibiting both MMP-2 and -9 expressions at 48 and 72 h. Considering the lack of certain data in literature on the activity duration of NAC molecule in cell culture studies, additional studies are needed to evaluate effects of different time points.

In the present study, Ca(OH)$_2$, only suppressed mRNA expression of MMP-2 and MMP-9 at 24 h. In light of these findings, the effectiveness of Ca(OH)$_2$, does not seem to prolong after 24 h. A previous study reported that Ca(OH)$_2$ induced MMP-2 expression and decreased cell viability in fibroblast cell culture after 24 h [19]. On the other hand, in teeth subjected to root canal treatment using Ca(OH)$_2$, as the root canal dressing, a lower inflammatory index was observed, accompanied by an increased proportion of fibroblasts and a decreased MMP-2, MMP-8 and MMP-9.
expression compared with single-visit root canal treatment in vivo [15]. Moreover, treatment of Prevotella nigrescens (P. nigrescens) LPS with Ca(OH)₂ resulted in down regulation of MMP-1 depending on different time intervals, whereas E. coli LPS treatment did not alter this gene expression levels in osteoblastic cell lines [14]. These differences in results might be attributed to differences in the experimental models.

In this study, 10 ng/mL E. coli LPS was used as bacterial stimulator. E. coli is the most common commercially found gram-negative bacteria, and its chemical structure and stimulatory effects have been extensively investigated [14]. It was reported that LPS induces strong inflammation by expression of proinflammatory cytokines and MMPs [23, 24]. Therefore, E. coli LPS was used to create effective inflammation in a representative model although it is not an actual periapical pathogen. However, mixed bacterial population in root canal infection may possibly be more complex than that observed in in vitro models. Additional studies are needed to evaluate the possible effects of various bacterial components on the destructive enzymes.

The results of our study showed that Ca(OH)₂ and NAC decreased the mRNA expression of TIMP-1 and TIMP-2 at 24 h, whereas NAC increased both TIMP-1 and TIMP-2 expressions at 48 h. It was reported that TIMPs have different cellular activities that seem to be independent of their primary inhibitory action on MMPs, which has not been detected yet [5]. On the other hand, in our study, increase of mRNA expression and protein levels of TIMP-1 were compatible with MMP-9 following NAC treatment. When MMP-9 expression increases, TIMP-1 expression may also increase to effectively inhibit the MMP-9 expression. However, a study revealed that Ca(OH)₂, effectively stimulated TIMP-1 mRNA expression with P. nigrescens and E. coli LPS stimulation depending on treatment duration and LPS concentration [14]. Such differences observed in different studies might be explained by the use of different cell lines and Ca(OH)₂ concentrations.

In conclusion, NAC, similar to Ca(OH)₂, decreased the gelatinolytic activity at 24 h. On the other hand, NAC seems to be more effective on TIMPs than Ca(OH)₂, and might be considered as an alternate candidate therapeutical agent to Ca(OH)₂. Additional studies are needed to evaluate the effectiveness of NAC molecules at higher pH with different endodontic pathogens.

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