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Original Article

Antimicrobial activity and biocompatibility of the mixture of mineral trioxide aggregate and nitric oxide-releasing compound

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KEYWORDS
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Abstract *Background/purpose:* Antimicrobial activity and biocompatibility of root canal sealer are related to the success of endodontic treatments. This study investigated the efficacy of mixture of mineral trioxide aggregate (MTA) and a NO-releasing compound for the antimicrobial activity, biocompatibility, and physical properties.
Materials and methods: MTA was mixed with diethylenetriamine-NO (MTA-NO), and the extracts from MTA and the MTA-NO mixture before and after setting was obtained were investigated the antimicrobial activity against *Enterococcus faecalis* and *Porphyromonas endodontalis*. After setting MTA and MTA-NO, pulp cell was incubated in the presence of MTA and MTA-NO disk using Transwell® cell culture insert, and the proliferation assay and mineralization-stimulated factors of the cells were analyzed by MTT assay and real-time RT-PCR, respectively. The physical properties of MTA and the MTA-NO mixture, such as surface hardness and flowability was also analyzed.
Results: The MTA-NO mixture showed stronger antimicrobial activity against *E. faecalis* and *P. endodontalis* than that by MTA. Both MTA and MTA-NO mixture increase the ratio of cell proliferation and induced the expression of alkaline phosphatase, collagen type I, osteocalcin, and osteopontin. Moreover, the induction of gene expression by MTA-NO mixture was higher than that by MTA alone. No significant difference was observed for surface hardness and flowability between MTA and MTA-NO mixture.
Conclusion: The addition of a NO-releasing compound to the endodontic treatment using MTA root canal sealer might reduce the risk of bacterial infection and help to regenerate the dental pulp tissue.

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Introduction

Control of bacterial infection into the root canal is closely related to the success of endodontic treatment.[1] Various studies have suggested five to seven bacterial species related to root canal infection,[2] and the most frequently isolated bacterial species from pulpitis being, *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, and *Enterococcus faecalis*.[3] *P. gingivalis* and *P. endodontalis* are associated with the initial infection of the root canal, and *E. faecalis* is closely related with apical periodontitis lesions of root canal-treated teeth.[4] Complete eradication of infectious bacteria in the root canal is difficult because the root canal system is anatomical complex. Therefore, root canal sealers have been developed using various chemical compounds to improve antimicrobial activity and biocompatibility.[5,6] Recently, mineral trioxide aggregate (MTA) has been used as an effective root canal sealer and filler.[7]

Several root canal sealers are developed and include bioceramic based sealers, calcium hydroxide, glass ionomer, silicone, resin, and zinc oxide eugenol.[6] Bioceramic based sealers are categorized into calcium silicate-based sealers and calcium phosphate-based sealers, and MTA belongs to calcium silicate-based sealer.[7] MTA is known as a bioactive endodontic cement owing to its biological, physicochemical and mechanical properties.[5,7] Even though compared to other sealers, MTA stimulates less inflammation and cell viability,[5] and tissue reactions such as collagen formation and fibroblast proliferation exhibited no difference.[8,9] Furthermore, compared to other sealers, MTA exhibited no remarkable antimicrobial activity against root canal infection related to bacteria.[4,10] Various studies have compared the antimicrobial activity of MTA and other sealers against general pathogens such as *Staphylococcus aureus* and *Escherichia coli*,[11,12] and MTA showed comparatively weak antimicrobial activity against *E. faecalis* than other pathogenic bacteria.[4]

Nitric oxide (NO) is hydrophilic, lipophilic, and a radical gas with antimicrobial activity.[13] NO exerts its antimicrobial activity against gram-positive and gram-negative bacteria by breaking down the bacterial wall and causing DNA damage.[14,15] In the human body, NO is synthesized by immune cells using NO synthase enzymes such as neuronal NOS, inducible NOS and endothelial NOS.[16] Since NO has a short half-life, it is synthesized as a delivery or releasing compound for use in chemotherapeutics and antimicrobial reagents.[17,18] Furthermore, NO binds to large immobile structures and reduce the toxic effect on the host tissue.[19] Among the NO-releasing compounds, diethylenetriamine (DETA) is a polyamine in eukaryotic and prokaryotic cells, and the antibacterial activity of DETA-NO against gram-negative and −positive bacteria have been previously reported.[17]

The present study investigated the antimicrobial activity of a mixture of MTA and NO-releasing compound against root canal infection related to bacteria, such as *E. faecalis* and *P. endodontalis*. Also, the physical properties between the MTA and MTA-NO mixture were compared to determine the efficacy of the methods.

Materials and methods

Preparation of MTA and MTA-NO mixture

White MTA base powder was purchased from Maruchi corp. and NO-releasing compound as diethylenetriamine-NO (DETA-NO; Sigmaaldrich Co., Saint Louis, MD, USA) was dissolved in sterilized distilled water to a final concentration of 1 M and 100 μl of DETA-NO solution was mixed with 100 mg of MTA. MTA and the MTA-NO mixture were dispensed into the inner wells of a 12-well polystyrene microplates (SPL LifeSciences, Gyeonggi, South Korea), and distilled water was dispensed in the outer wells of the microplate to maintain a stable humidity level. The sealers were set at 37 °C for 24 h. The MTA disks were immersed in 2 ml of phosphate buffered saline (PBS, pH 7.2) and the cell culture media for susceptibility assay and bioactivity, respectively. Each eluate was transferred into a fresh 15 ml conical tube and centrifuged at 4000×*g* for 10 min to remove any remaining particles.

Bacterial strain and cultivation

E. faecalis ATCC 29212 was cultivated in brain heart infusion (BHI) broth (BD Biosciences, Sparks, MD, USA), and *P. endodontalis* ATCC 35406 was cultured in BHI broth supplemented with hemin (1 μg/ml) and vitamin K (0.2 μg/ml) at 37 °C in an anaerobic condition (5% H_2, 10% CO_2, 85% N_2).

Antimicrobial activity

The susceptibility assay of *E. faecalis* and *P. endodontalis* for eluates was performed according to the protocols of Clinical Laboratory Standard Institute (CLSI). A suitable medium (180 μl) was dispensed into a 96-well plate (SPL Lifescience), and the eluate of MTA and MTA-NO mixture was added into the 12th row well of the broth-contained wells. The eluates (180 μl) to the 2nd row were 2-fold serially diluted using a multi-pipette. The concentration of the cultured bacteria was adjusted at 1.0×10^6 cells/ml for *E. faecalis* and 1.5×10^6 cells/ml for *P. endodontalis* after counting the bacteria with bacteria counting chamber (Marienfeld, Lauda-Konigshöfen, Germany). Twenty microliters of the bacterial suspensions was inoculated into the well containing the prepared broth, and the plate was incubated at 37 °C under anaerobic condition (H_2 5%, CO_2 10%, N_2 85%) for 36 h. The optical density for the microplate was measured using a spectrophotometer (Biotek,

Winooski, VT, USA) at 660 nm of wavelength to determine the growth of *E. faecalis* and *P. endodontalis*.

Cell culture and treatment

Human pulp cells were purchased from AXOL Bioscience (Little chesterford, Cambridge, UK) and cultivated with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin; Hyclone) for 7 to 10 passages. The cells were seeded on a 12-well plate (SPL Lifesciences). When pulp cells reached 80% confluence, the medium was removed, and fresh medium without FBS was added to starve the cells. After incubating for 4 h, the medium was removed again, and Transwell® cell culture inserts (pore size of 3.0 μm) (Corning Inc., Lowell, MA, USA) were hung in the wells of the 12-well plate. The setting MTA and MTA-NO disk (5 mm of diameter x 2 mm of thickness) were placed into the cell culture insert, and 1.5 ml and 0.4 ml of fresh medium were added into the plate well and the cell culture insert, respectively. The plate was incubated in a 5% CO_2 for 24 h. The cells were used for RT-PCR.

Cell proliferation assay

Human pulp cell was seeded in 12-well polystyrene plate (SPL Lifesciences) at a density of 5×10^3 cells/well. After incubating for 6 h, the medium was removed, and the cell culture inserts were hung in the well. MTA and MTA-NO disk (5 mm of diameter x 2 mm of thickness) were placed in the cell culture insert, and the fresh medium were then added into the plate well and the cell culture insert. The images of the cells were taken at time point 0, and after 24 h and 48 h of treatment using phase contrast microscope (Olympus CKX53, Tokyo, Japan). Also, the cell was treated with MTA and MTA-NO mixture at three time points, and the cell culture medium was then removed using suction pump. The cells were treated with 4 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 4 h in 5% CO_2 incubator. After washing the cell with phosphate buffered saline (pH 7.2),

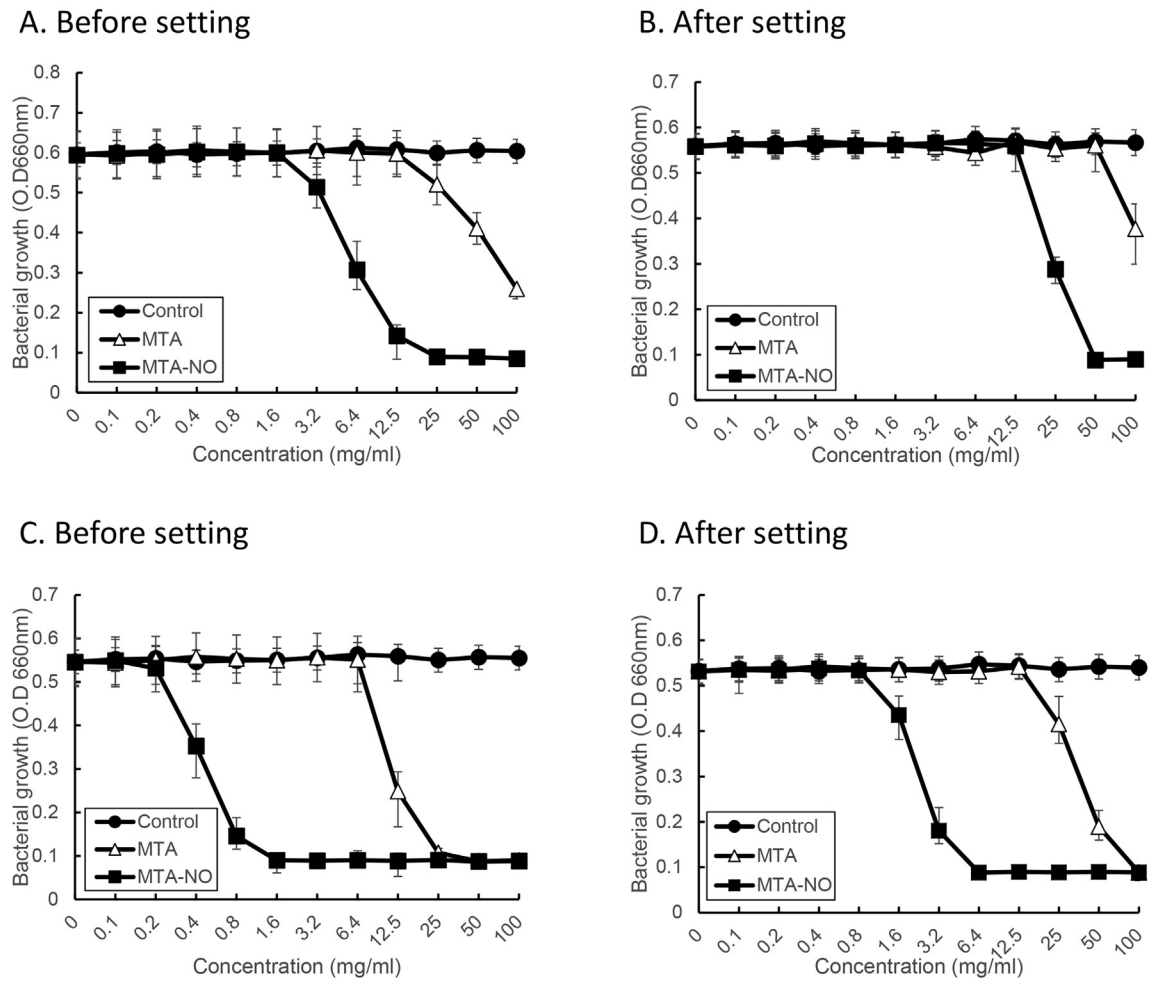

Figure 1 Antimicrobial activity of the extracts from MTA and MTA-NO mixture before and after setting. *E. faecalis* (A and B) and *P. endodontalis* (C and D) were cultured with the extracts from MTA and MTA-NO before and after setting in a 96-well microtiter plate. Bacterial growth was measured by a spectrophotometer at 660 nm of wavelength after cultivating for 36 h. The experiments were performed three times in triplicate, and the data are represented as mean and STDEV. Asterix (*) indicates statistically significant difference compared with the control group ($P < 0.05$).

the formazan crystals in the cells was dissolved with Ethyl alcohol for 10 min using a rocker. The formazan product was measured with a spectrophotometer at a 540 nm of wavelength to analyze live cell density.

Real-time reverse-transcription polymerase chain reaction

The pulp cells were washed with cold PBS, and total RNA from the cells was isolated with TRIzol® reagent (Invitrogen Life Tech, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA from the isolated total RNA (1 μg) was synthesized by a Maxime™ RT Premix (iNtRON, Gyeonggi, Korea) containing oligo primer. The cDNA samples were mixed with 25 μl of SYBR Premix Ex Taq (TB GreenTM Premix Ex TaqTM II; Takara, Tokyo, Japan), 0.4 μM of each primer, and ROX dye, and the mixture was adjusted to 50 μl of final volume with distilled water. The prepared samples were performed semi-quantitative polymerase chain reaction by ABI 7500 Real-Time PCR system (Applied Biosystems, Foster, CA, USA). The PCR thermal protocol consisted of a 4 min 95 °C denaturation step, followed by 40 cycles of a 15 s at 95 °C, a 15 s at 60 °C and a 33 s at 72 °C. The sequences of each primer were as follows: 5′-GGA CCA TTC CCA CGT CTT CAC-3′ and 5′- CCT TGT AGC CAG GCC CAT TG-3′ for alkaline phosphatase (ALP) gene; 5′-CTG GCA CAG GGT ATA CAG GGT TAG-3′ and 5′-ACT GGT GCC GTT TAT GCC TTG-3′ for bone sialoprotein (BSP) gene; 5′-CTG CTG GAC GTC CTG GTG AA-3′ and 5′-ACG CTG TCC AGC AAT ACC TTG A-3′ for collagen type I (Col I) gene; 5′- CGG TGC AGA GTC CAG CAA AG-3′ and 5′- TAC AGG TAG CGC CTG GGT CT-3′ for osteocalcin (OC) gene; 5′- ACA CAT ATG ATG GCC GAG GTG A-3′ and 5′- GTG AGG TGA TGT CCT CGT CTG TAG-3′ for osteopontin (OPN) gene; 5′-CAC TGG CGC TGC AAC AAG A-3′ and 5′-CAT TCC GGA GCT CAG CAG AAT AAT-3′ for Runx2 gene; 5′-GTG GTG GAC CTG ACC TGC-3′ and 5′-TGA GCT TGA CAA AGT GGT CG-3′ for the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The house keeping gene as GAPDH was used as a reference to normalize the expression levels to quantify changes in the target gene expression. The PCR products were analyzed for each amplification product using a dissociation curve of amplification.

Physical properties

The physical properties, such as surface hardness and flowability of MTA and MTA-NO mixture were analyzed. The surface hardness of the samples was analyzed by Vickers hardness testing machine (HM-200, Mitutoyo, Kawasaki,

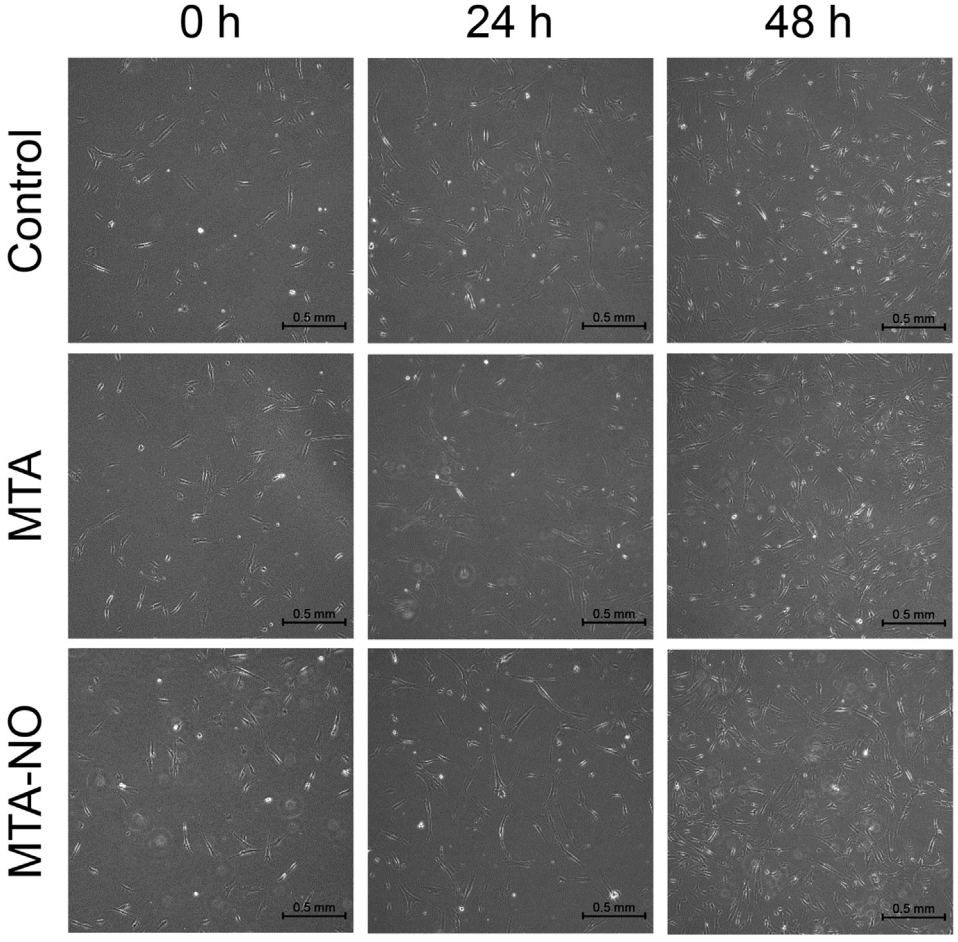

Figure 2 Cell proliferation. Pulp cells were incubated in the presence or the absence of MTA and MTA-NO disk using Transwell® culture insert for 24 h and 48 h. the cell image were taken by a phase contrast microscope.

Japan). The samples were loaded at 500 gf for 20 s using a pyramid-shaped diamond indenter, and the diagonal of the resulting indentations was measured using a microscope. The Vickers hardness values displayed on the digital readout of the machine were recorded. The surface hardness was measured by three indentations on the surface of the samples at separated locations.

Flowability test was performed as recommended by ANSI/ADA 57—2000. Briefly, MTA pastes and MTA-NO mixture were placed on a flat glass slab. After 3 min, another glass slab was placed over the samples. A mass of 120 g was placed on top of the overlying glass slab, and the set up was kept in an incubator (37 °C and 95% humidity using pre-warmed distilled water) for 10 min. The weight was removed, and a digital caliper measured the maximum and minimum diameters of the circular shape formed by the samples. When the difference between the maximum and minimum diameters did not exceed 1 mm, the test was repeated. The average value of both diameters was recorded as the measurement of flowability for each sample.

Statistical analysis

All the experiments were repeated three times in three sets independently, and IBM SPSS Statistics Ver. 23 (IBM, Armonk, NY, USA) was used for statistical analysis. The data were evaluated for normal distribution by Kolmogorov—Smirnov test. The values among groups were analyzed by a nonparametric Kruskal—Wallis test and Mann—Whitney test. Statistical significance was defined by a P-value of less than 0.05. All data were expressed as median and interquartile range.

Results

Antimicrobial activity of MTA and MTA-NO mixture

The growth of *E. faecalis* was significantly inhibited in the elute of MTA and MTA-NO mixture before setting above the concentration of 50 mg/ml and 6.4 mg/ml, respectively ($P < 0.05$) (Fig. 1A). Also, the elute of MTA and the mixture after setting showed antimicrobial activity against *E. faecalis* at a concentration above 25 mg/ml and 100 mg/ml, respectively (Fig. 1B). In case of *P. endodontalis*, the elute of MTA and MTA-NO mixture before setting significantly inhibited bacterial growth above the concentration of 12.5 mg/ml and 0.4 mg/ml, respectively ($P < 0.05$) (Fig. 1C). Also, the elute of MTA and MTA-NO mixture significantly reduced the growth above the concentration of 25 mg/ml and 3.2 mg/ml, respectively ($P < 0.05$) (Fig. 1D). A comparison of the antimicrobial activity between MTA and MTA-NO mixture showed that MTA-NO mixture had a stronger and significant the antimicrobial activity against *E. faecalis* and *P. endodontalis* than that by MTA.

Biocompatibility of MTA and MTA-NO

When the proliferation of pulp cell was observed in the presence and the absence of MTA and MTA-NO disk, more cells were observed in the presence of MTA and MTA-NO disk (Fig. 2). Furthermore, in MTT assay, the cell proliferation significantly increased in the presence of MTA and MTA-NO disk compared to control ($P < 0.05$) (Fig. 3) and the difference between MTA and MTA-NO disk did not exhibit significantly. Pulp cell was incubated in the presence of MTA and MTA-NO disk for 24 h, and the induction of mineralization-related genes was investigated. The expression of mineralization-related genes, such as *ALP, BSP, Col I, OC, OPN* and *Runx2* in pulp cell was significantly induced by MTA and MTA-NO disk compared to that in the control cells ($P < 0.05$) (Fig. 4). Of these, three genes (ALP, OC, and Runx2) were induced more prominently by MTA-NO disk than by MTA disk. ($P < 0.017$). Otherwise, the induction of the other genes, such as *BSP, Col I*, and *OPN* showed no significant difference between MTA and MTA-NO disk treatment.

Analysis of physiological properties

Analyzing surface hardness between setting MTA and MTA-NO using a Vickers microhardness testing machine, the surface hardness of MTA and MTA-NO showed 7.455 ± 0.1625 HV and 7.335 ± 0.1225 HV, respectively (Table 1). Also, there was no significant difference between the two groups. Similarly, the flowability of MTA and MTA-NO showed no significant difference with a median of 21.145 ± 0.407 mm and 20.91 ± 0.502 mm, respectively (Table 2).

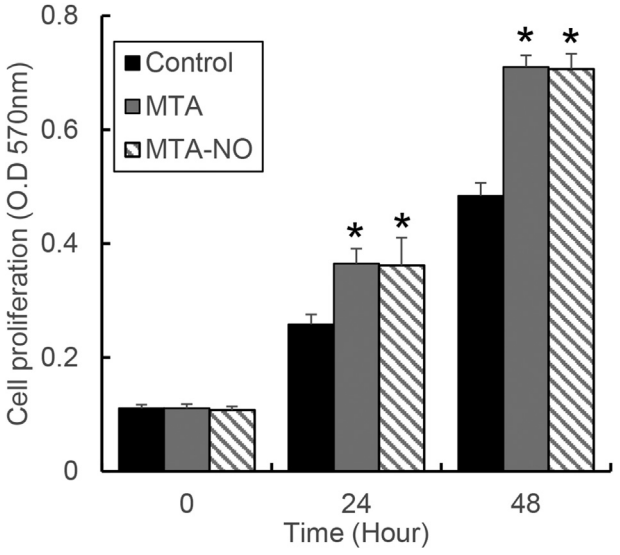

Figure 3 Cell proliferation assay. Pulp cells were incubated in the presence or the absence of MTA and MTA-NO disk using Transwell® culture insert for 24 h and 48 h. After removing the media, MTT solution was added in the well containing cell, and the cells were incubated for 4 h. The optical density was measured by a microplate reader. The experiments were performed three times in duplicate, and the data are represented as mean and STDEV. Asterix (*) indicates statistically significant difference compared with the control group ($P < 0.05$).

Discussion

Various materials have used to develop root canal sealers, such as epoxy resin, zinc oxide, and calcium hydroxide.[6,8,20] Recently, MTA, a bioceramic sealer, has been widely used as root canal sealer, owing to its physical and biological properties.[21,22] Previous studies have reported the role of NO in osteoblast differentiation, regulation of inflammation, and bactericidal activity.[13,23,24] Therefore, the present study was investigated the biocompatibility, antimicrobial activity, and physical properties of MTA root canal sealer with or without a NO-releasing compound.

The extract from MTA and MTA-NO mixture before or after setting was examined for the antimicrobial activity, and the extract from MTA-NO mixture showed stronger antimicrobial activity against *E. faecalis* than MTA alone. Interestingly, the antimicrobial activity of MTA showed stronger against *P. endodontalis* as a gram-negative bacterium than *E. faecalis* as a gram-positive bacterium. Among components of MTA, metal oxides have antimicrobial activity through destabilizing bacterial membrane and binding to the cytosolic components of bacteria.[25] Therefore, the presence of abundant peptidoglycan in gram-positive bacteria weakens the antimicrobial activity of metal oxides. NO may complement this phenomenon by action of bacterial DNA damage after penetration of the bacterial wall.[13] Based on this mechanism, when MTA and NO are presented together, the synergistic effects of the two molecules might result in a stronger antimicrobial activity.

The biocompatibility of MTA and MTA-NO mixture was investigated. The biocompatibility was analyzed using cell proliferation and mineralization-related factors. When the cell proliferation was investigated using MTT assay in the presence of the absence of MTA and MTA-NO disk, two disks increased the ratio of the cell proliferation compared to control. Next, the change of mineralization-stimulated factors by MTA and MTA-NO was investigated. The expression of mineralization-related factors, such as ALP, Col I, OC and OPN was more induced in MTA and MTA-NO disk treated cells than in the control group (non-treated cells). Furthermore, the increase in expression of the mineralization-related factors was higher in the presence of MTA-NO disk than MTA disk, suggesting the role of NO in inducing the gene expression. The effect of NO on the biocompatibility of osteoblast cell is dose dependent,[23] and a high concentration of NO reduced cell viability of human gingival fibroblast,[26] and inhibited proliferation of osteoblast.[27] However, low concentration of NO induced the expression of mineralization-stimulated factors, such as

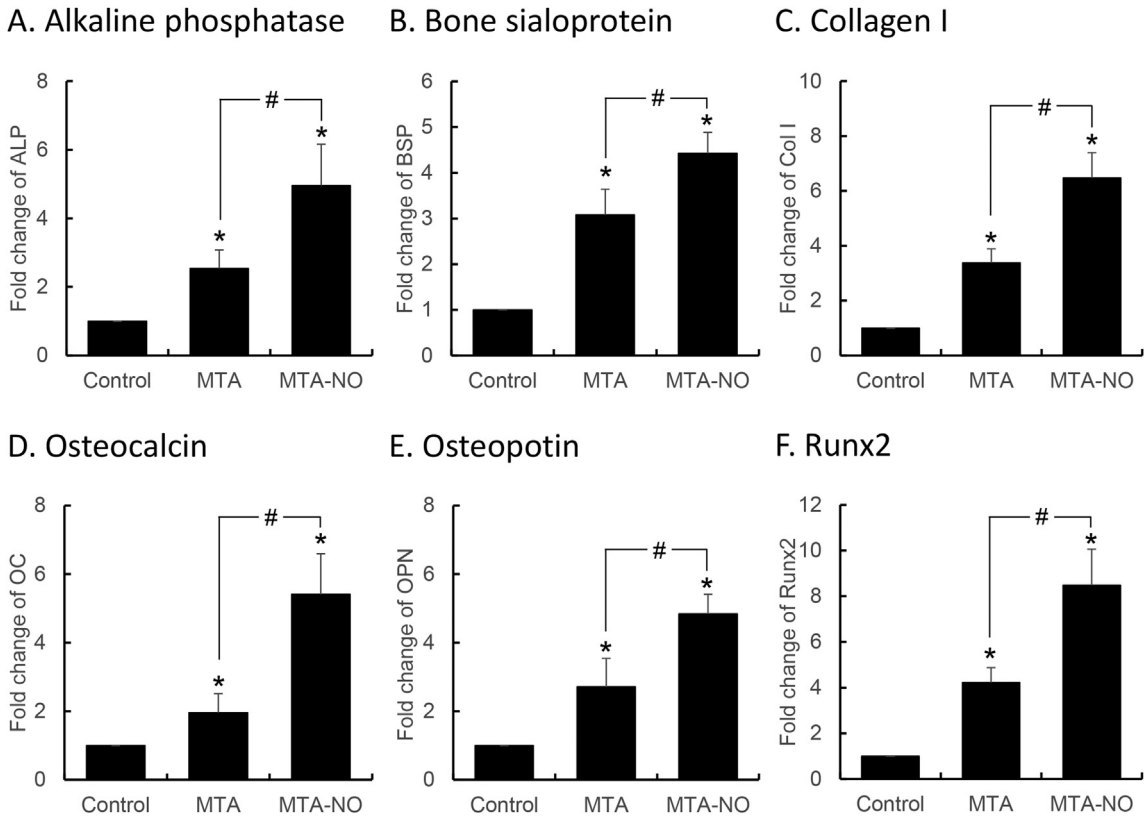

Figure 4 Induction of the expression of mineralization-related genes by MTA and MTA-NO mixture. Human pulp cells were cultured in the presence or absence of MTA and MTA-NO disk using Transwell® culture insert for 24 h. After extracting total RNA from the cultured cells, the expression of ALP (A), Bone sialoprotein (B), Col I (C), OC (D), OPN (E), and Runx2 (F) was analyzed by real-time RT-PCR. The experiments were performed three times in duplicate, and the data are represented as median and interquartile range. Asterisk (*) indicates statistically significant differences compared with untreated cells ($P < 0.05$), and sharp (#) indicates statistically significant differences between MTA and MTA-NO treated cells ($P < 0.05$).

Table 1 Surface hardness of mineral trioxide aggregate (MTA) and MTA-nitric oxide (MTA-NO) disk.

Samples	Mean (HV)	STDEV (HV)
MTA disk	7.455	0.1625
MTA-NO disk	7.355	0.1225

Data presented as mean and standard deviation (STDEV).
HV: Vickers pyramid number (kgf/mm^2).

Table 2 Flowability of MTA and the mixture of MTA and NO.

Samples	Mean (mm)	STDEV (mm)
MTA disk	21.145	0.407
MTA-NO disk	20.910	0.502

Data presented as mean and standard deviation (STDEV).

ALP, OPN, and Runx2.[28] Moreover, NOC-18, NO releasing compound, reduced the growth of dental pulp cells and enhanced the mineralization and apoptosis of pulp cells.[29]

Utilization of the MTA-NO for clinical treatment requires the mixture to have the same or better physical properties than those by MTA alone. Therefore, the physical properties of MTA and MTA-NO mixture were analyzed. Since, flowability plays an important role in adaptation to various irregularities present in the root canal system and improves the ability of the material to permeate into perforations,[30] this study examined the flowability of MTA-NO mixture. The flowability of MTA-NO mixture did not show the significant difference compared to the flowability of MTA. In addition, the surface hardness of MTA-NO disk did not exhibit a significant difference compared to that of MTA-disk. Microhardness is possible to be used as an indicator of completeness of the setting reaction.[15] Taken together, MTA-NO mixture might have physical properties similar to those of MTA.

In conclusions, when a NO-releasing compound, as DETA-NO, is mixed with MTA powder, NO is released rapidly from DETA-NO in the initial phase or before setting of the mixture, and the process becomes slower after setting. Therefore, in the early stage, the mixture of MTA and NO has potent antimicrobial activity due to the mineral oxide and high concentration of NO and might prove effective in removing oral microbes. Moreover, the low concentration of NO as NO released from the mixture might help in the bone generation and wound healing after endodontic treatment.

Declaration of competing interest

The authors have no conflict of interest to declare.

Acknowledgments

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