N-acetylcysteine attenuates PGE\textsubscript{2} and ROS production stimulated by 4-META/MMA-based resin in murine osteoblastic cells

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This study examined the effects of N-acetylcysteine (NAC) on the inflammatory reactions of murine osteoblastic cells cultured on the 4-methacryloxyethyl trimellitate anhydride/methyl methacrylate (4-META/MMA)-based resin. Superbond C&B (SB) was used as the 4-META/MMA-based resin and placed in a 48-well cell culture plate. The cells were cultured in αMEM (control) as well as on SB and SB in αMEM with NAC (SB+NAC). They were examined using the WST-1 proliferation assay, real-time PCR, enzyme-linked immunosorbent assay (ELISA), intracellular reactive oxygen species (ROS) measurements, and cellular glutathione (GSH) detection. COX-2 and IL-6 gene expressions were upregulated in SB; however, they were suppressed by NAC. Furthermore, PGE\textsubscript{2} production in the culture medium was increased in SB, whereas NAC decreased the PGE\textsubscript{2} production. NAC lowered the ROS level in the culture medium and significantly increased the intracellular GSH level. The present in vitro study demonstrated that NAC might be effective for dental material detoxification.

Keywords: 4-META/MMA-based resin, N-acetylcysteine, Reactive oxygen species

INTRODUCTION

Vertical root fracture (VRF) in teeth has poor prognosis and necessitates tooth extraction in many cases\textsuperscript{1}. Some studies have reported an alternative method of intentional replantation of teeth with VRFs after bonding using the 4-methacryloxyethyl trimellitate anhydride/methyl methacrylate (4-META/MMA)-based resin as an adhesive to preserve the vertically fractured tooth\textsuperscript{2,3}. The prognosis is generally improved by performing intentional replanting of the tooth with VRF. However, some studies have shown that the 4-META/MMA-based resin induces an inflammatory reaction\textsuperscript{4,5}. Moreover, the 4-META/MMA-based resin causes potentially adverse biological effects, such as degeneration of the pulp and periodontal tissues\textsuperscript{6}. The inflammatory reaction of tissue to the 4-META/MMA-based resin is a clinical concern. The resin can be modified to improve its biological properties. Resin monomers decrease the intracellular antioxidant activity by generating reactive oxygen species (ROS)\textsuperscript{7}. Apoptosis is strongly induced by oxidative stress, which is disproportionate to ROS and the antioxidant ability\textsuperscript{8}. Specifically, the ROS induce oxidative damage, resulting in significant cell loss.

N-acetylcysteine (NAC) is an amino acid that is indicative of the intracellular antioxidant activity. NAC prompts the production of a cellular antioxidant molecule, glutathione (GSH)\textsuperscript{9}. Currently, NAC is primarily used as an antidote for acetaminophen overdose\textsuperscript{10}, to prevent nephropathy associated with radiographic contrast media\textsuperscript{11}, and as a mucolytic substance\textsuperscript{12}. Additionally, NAC can directly scavenge ROS because it comprises a sulfhydryl group that exhibits a strong reduction ability\textsuperscript{13}. Therefore, we hypothesized that NAC could have a potential protective action against the effects of the 4-META/MMA-based resin. The purpose of this study was to examine whether NAC detoxified and prevented inflammatory reactions in cells cultured on the 4-META/MMA-based resin.

MATERIALS AND METHODS

Preparation of 4-META/MMA-based resin

Super bond C&B (SB; Sun Medical Co., Ltd., Shiga, Japan) was used as the 4-META/MMA-based resin. The specimens were prepared according to the instructions provided by the manufacturer. The powder, monomer, and catalyst (four drops of liquid monomer, one drop of catalyst, and 0.08 g of polymer powder, respectively) were placed in a 48-well cell-culture-grade polystyrene dish and subsequently mixed. The specimens were then allowed to set for 72 h at 37°C in a humidified 95% air atmosphere with 5% CO\textsubscript{2}.

Cell culture

Murine osteoblastic MC3T3-E1 cells (Riken Cell Bank no. RCB1126, Tsukuba, Japan) were grown in alpha-modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 284 µM L-ascorbic acid 2-phosphate, and 66.7 µg/mL kanamycin sulfate. MC3T3-E1 cells were grown at 37°C in a humidified atmosphere of 95% air with 5% CO\textsubscript{2}, and the cells were cultured on SB in a 48-well culture dish at a density of 3×10\textsuperscript{4} cells/cm\textsuperscript{2}. The pH
of the NAC solution was adjusted to 7.2 to ensure that it had the density of 1 mol/L. The cells were cultured on SB with or without 20 mM NAC.

**WST-1 proliferation assay**

A WST-1 assay (Roche Applied Science, Mannheim, Germany) was used to evaluate cell proliferation. The cells were cultured on SB alone, SB in αMEM with NAC (SB+NAC), and polystyrene αMEM (control) for 12–48 h (n=8).

**Quantitative real-time PCR**

MC3T3-E1 cells were cultured on SB at a density of 3×10⁴ cells/cm² with and without the addition of 20 mM NAC for 24 h. Total ribonucleic acid (RNA) was extracted from the cells using Trizol (Invitrogen). cDNA was synthesized from 1 µg of the total RNA using ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan) and oligo dT primers (TOYOBO).

A quantitative real-time polymerase chain reaction (PCR) was performed using ABI 7300 (Applied Biosystems, Foster city, CA, USA) to detect the PTGS2 (COX-2) and interleukin-6 (IL-6) mRNA. The specific primer sets used for PTGS2 (Mm00478374_m1), IL-6 (Mm00446190_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm99999915-g1) were designed using the Primer Express program (Applied Biosystems).

The comparative Ct method was used to calculate the mRNA expression. The comparative Ct method is also known as the 2⁻ΔΔCt method, where ΔΔCt=ΔCt (sample)−ΔCt (reference). ΔCt is the difference in the threshold cycle between the samples of interest and the GAPDH control.

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

The PGE₂ levels secreted from the cells attached on SB treated with or without NAC were analyzed immunochemically at 48 h by using the PGE₂ high-sensitivity ELISA kit (Enzo Life Science, NY, USA), according to the instructions of the manufacturer (n=8).

**Intracellular ROS production**

The quantity of intracellular ROS production at 24 h was measured using fluorometry with 2,7-dichlorofluorescin diacetate (DCFH-DA) (Invitrogen). After removing the supernatant, the cells were rinsed in D-PBS and active 0.25% trypsin-1 mM-EDTA-4Na. After centrifugation, the cells were incubated with D-PBS containing 20 µM DCFH-DA solution for 15 min at 37°C. The fluorescence intensity of DCF-DA was measured using Wallac ARVO SX 1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA) at 535 nm (excitation at 485 nm). The ROS levels for the cells cultured on polystyrene (cells without any treatment) were considered to be 100% (n=8).

**Measurement of cellular glutathione**

A total glutathione quantification Kit (Dojindo Molecular Technologies, Rockville, MD, USA) was used to measure the quantity of cellular glutathione. The cells were incubated for 24 h; subsequently, the culture medium was removed. Next, we added hydrochloric acid to destroy the cell membrane and cultured it with DTNB ([5,5ʹ-dithiobis(2-nitrobenzoic acid)) and glutathione reductase for 10 min at 37°C. The cellular GSH concentrations were determined by measuring the absorbance at 415 nm.

**Statistical analysis**

The statistical significance of differences among the results was analyzed using ANOVA with a post hoc Bonferroni test at an α level of 0.05.

**RESULTS**

**NAC alleviates cell reduction on SB**

We examined the cell proliferation on SB after 24 h of incubation. In addition, we determined whether the cell proliferation on SB improved when using a predetermined NAC concentration. It was observed that SB induced cell reduction, which was recuperated by NAC. There were no significant differences between the control and 20 mM NAC at 48 h (Fig. 1).

**NAC reduces COX-2 and IL-6 gene expression**

To evaluate the effect of NAC on the mRNA expression of the gene associated with inflammatory cytokine expression, the cell cultured for 24 h was analyzed for the mRNA expression of COX-2 and IL-6 genes using real-time PCR analysis. The COX-2 mRNA expression was 5-fold upregulated on SB than on the control. Additionally, the IL-6 gene expression was over 8-fold upregulated on SB than on the control. However, NAC reduced the increase in the COX-2 and IL-6 gene expressions induced by SB (Fig. 2).

**NAC reduces secretion of PGE₂ in cell culture on SB**

To test the effects of NAC on the production of proinflammatory molecules, the amount of PGE₂ in the culture medium was measured by ELISA. SB induced a marked increase in the PGE₂ production in the culture medium. However, NAC decreased the PGE₂ production induced by SB (Fig. 3).

**Suppression of ROS production of cells on SB**

To determine whether SB induced cytotoxicity owing to the production of ROS, the effect of NAC on the ROS levels was investigated. The ROS levels measured using DCFH-DA revealed that treatment with NAC attenuated the ROS production of cells on SB (Fig. 4A).

**NAC enhances the antioxidant capacity of cells on SB**

The amount of intracellular GSH after 24 h is presented in Fig. 4B. Although the amount of GSH was reduced by SB, it was increased significantly by NAC.
DISCUSSION

SB was developed approximately 40 years ago and has since then been widely used as luting cement\(^{14}\), root canal sealer\(^{15}\), and in dental treatments such as intentional replantation\(^{16}\). In previous studies, SB exhibited cytotoxicity induction in human pulp fibroblasts\(^{5}\) and inhibition of osteogenic activity in rabbit dental pulp tissue\(^{17}\). Particularly, the cytotoxicity of SB is greatest over the initial days and then decreases\(^{18}\). In this study, SB inhibited the proliferation of MC3T3-E1 cells. The inhibition was alleviated by 20 mM NAC (Fig. 1), which suggested that NAC could control the early stage cytotoxicity of SB.

Cell death is affected by various factors inside and outside the cell. Particularly, a significant increase in ROS induces apoptosis by the activation of apoptosis signal-regulating kinase 1\(^{19}\). The antioxidant action of GSH regulates cell proliferation and death, which is induced by the oxidative process\(^{20}\). In this study, SB induced the production of ROS in MC3T3-E1 cells, which suppressed cell proliferation; moreover, the reduction in GSH was related to this phenomenon (Fig. 4).

This study demonstrated that NAC suppressed COX-2 expression and inhibited the PGE\(_2\) production in the cells on SB. ROS induce COX-2 expression and subsequent PGE\(_2\) production\(^{21}\). IL-6, a pro-inflammatory cytokine, plays an important role in the regulation of acute and chronic inflammation\(^{22}\). NAC inhibits the IL-6 mRNA expression, while possibly attenuating the effect of the anti-inflammatory action via IL-6 mRNA expression. Cox-2 is responsible for elevated levels of PGE\(_2\), which is a pro-inflammatory mediator. During
inflammation, the ROS play an important role in mediating various signal transduction cascades. The results of this study show that NAC can reduce the production of PGE₂, which is an important chemical mediator for inflammation. Therefore, the results of this study suggest that NAC regulates PGE₂ production by the sequential activity of COX-2. Moreover, NAC suppresses the IL-6 production induced by lipopolysaccharide stimulation, and down-regulates JNK and p38 MAPK activation. A transcription factor, nuclear factor-kappa B (NF-xB), plays an important role in controlling inflammation by regulating COX-2. Amore et al. reported that NAC resets the activation of NF-xB to normal values in the lymphomonocytes of diabetic hemodialysis patients. Further study is needed to confirm the influence of NAC on the COX-2 expression via the NF-κB pathway.

The effect of NAC varies according to the density. At concentrations exceeding 25 mM, NAC activated alkaline phosphatase and induced mineralization in the cultured osteoblastic cells, whereas 5 mM NAC strongly inhibited TGFβ1-induced nuclear translocalization of the p65 NF-κB subunit and decreased the ROS production. The NAC density of 20 mM used in this study may induce calcification; however, further study is needed to confirm whether 20 mM NAC induces the calcification of MC3T3-E1 cells.

The survival rate of teeth subjected to intentional replantation three years ago has been reported to vary from 70 to 90%. However, the rate is influenced by material biocompatibility. Additionally, the application of NAC may improve the prognosis of intentional replantation. Further in vivo studies will be necessary to examine these results in detail.

CONCLUSION

The present in vitro study demonstrated that the 4-META/MMA-based resin induced the production of PGE₂ and ROS. NAC attenuated these effects and reduced the proinflammatory cytokine mRNA expression. The current findings can be applied to dental materials. As a scope for future studies, investigation of the effects of NAC on the dental pulp is necessitated.

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CONFLICT OF INTEREST

None.

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