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N-Acetyl cysteine prevents suppression of oral fibroblast function on poly(methylmethacrylate) resin

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Abstract

Despite the proven cytotoxicity, poly(methylmethacrylate) (PMMA) resin is one of the most frequently and extensively used materials in medical and dental fields. The study examined the potential detoxification of the resin material and restoration of the resin-induced suppression of cellular function using an antioxidant amino acid derivative, *N*-acetyl cysteine (NAC). Oral fibroblasts extracted from rat oral mucosa were cultured on the resin material with or without incorporation of NAC into the material. Twenty-four hour after incubation, less than 2% of the cells were viable on the untreated control resin, while up to 35% of the cells were viable on the resin with incorporation of NAC. At day 7 of culture, the expression of collagen I and III genes was downregulated on the untreated resin, while the cells on NAC-supplemented resin showed the expression levels similar to those in polystyrene culture. The cells produced three times greater amount of collagen on the NAC-supplemented resin than on the untreated resin. The data demonstrated that the cytotoxicity of PMMA resin was substantially lower when the material contains NAC. The potential usefulness of this principle should be explored with a view of developing biocompatible polymer-based materials in a broad range of dental and medical resin materials and tissue engineering scaffolds.

Keywords: Dental resin; Bone cement; Cytotoxicity; Fibroblasts; NAC

1. Introduction

Resin-based materials are used for many applications in dental and medical fields. In addition to the physical and chemical properties, the clinical success of the materials depends also on their biological safety. Among these materials, acrylic-based self-polymerizing dental resin, which primarily consists of a solid part of prepolymerized poly(methylmethacrylate) (PMMA) and a liquid part of methyl methacrylate (MMA), is considered as one of the most frequently used resin materials in daily dental practice. It has been used successfully for the fabrication of temporary crowns, denture base and temporary seal of prepared cavities. However, its biological safety has been a major concern [1]. MMA monomer is well known to cause allergic and/or toxic reactions [2]. Incomplete polymerization of PMMA dental resin under clinical conditions results in unreacted monomers that may be released from the resin matrix into the aqueous environment of oral cavity [3]. Such release of monomers is also observed even after complete polymerization of the resin [4]. Oral hypersensitivity reactions and local irritation caused by MMA and general toxicity and sensitivity to MMA have been reported [1,2]. Fibrosis, necrosis and histocytosis are found locally in tissues around resin material [5,6].

At the cellular level, MMA monomer has been identified as cytotoxic by a variety of different methods, all indicating changes in basic cell structures, such as cell membrane integrity and cell functions like enzyme activities or the synthesis of macromolecules [7–9]. Although still not clear, the mechanisms of adverse effects caused by MMA monomer are thought to involve direct toxicity from released or

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residual MMA and oxidative stress created by free radicals that are released during the resin polymerization [10,11].

Fibroblasts constitute the predominant cell type of oral connective tissue, which contains both gingival and periodontal ligament fibroblasts. The preservation of integrity and health of these cells is essential for healthy related tissues. Studies utilizing permanent cell lines or primary culture of fibroblasts from various origins such as dental pulp, gingival and periodontal ligament demonstrated mutagenicity and cell death by exposure to MMA [7,9,12-14]. Therefore, the development of a biocompatible PMMA resin can help to overcome such adverse effects to fibroblasts.

The glutathione (GSH) is a cysteine derivative, and the glutathione mediated redox cycle is considered as the most important removal system for free radicals [15,16]. *N*-Acetyl cysteine (NAC) is a pro-GSH drug. It is easily deacetylated into L-cysteine, which is an important precursor of glutathione [17], and helps promote the cellular glutathione system [18,19]. It also acts as a direct strong oxidant scavenger [20]. It was therefore hypothesized that NAC has detoxifying and protective effects for fibroblasts against PMMA-based dental resin, which, if proven, might provide a novel solution to overcome the adverse effects of the PMMA-based resin.

The purpose of this study was to examine cell viability and function of oral fibroblasts when cultured on a PMMA-based auto-polymerizing resin material. Also, it was determined whether the impaired viability and function of the cells can be prevented on the material containing NAC.

2. Materials and methods

2.1. Resin preparation

Untreated control self-polymerizing dental resin (Unifast II, GC, Tokyo, Japan) was prepared by mixing the powder and liquid components (Table 1) for 15 s according to the manufacturer's recommendations (powder/liquid ratio of 0.6/0.4 g; total weight 1.0 g well^{-1}) in a well (22 mm in diameter) of a 12-well cell culture-grade polystyrene dish. The resin mixture was spread over the well and the surface of the resin was leveled by vibrating the culture dish during its initial polymerization stage. The experimental NAC-supplemented resin was prepared by mixing the powder and liquid containing various concentrations of NAC (0.15%, 0.4% or 0.6% in weight percent of the final resin substrate). The concentration of NAC was determined by our previous study, in which PMMA-suppressed phenotypes of rat dental pulp cells were restored in the NAC-dose dependent manner [21]. The mixed resin was allowed to polymerize in the well at 37 °C for 30 min and rinsed with dd H_2O once before seeding cells.

Table 1
Formulation of self-curing PMMA resin

Powder	Ethyl–methyl copolymer	70 wt.%
	Polymethylmethacrylate	30 wt.%
	CEBA	1.0 phr
	ACu	10 ppm
Liquid	Methylmethacrylate	95 wt.%
	Ethyleneglycol dimethaclylate	5 wt.%
	LMAC	1.0 phr

CEBA, 1-cyclohexyl-5-ethyl barbituric acid; ACu, acetylacetone copper; LMAC, dilauryl dimethyl ammonium chloride.

2.2. Oral fibroblast cell culture

Fibroblasts were obtained from palatal tissue of 8-week-old Sprague–Dawley rats. After sacrificing the animals, the palatal tissue was aseptically removed and washed with 1% phosphate buffered solution (PBS, MP Biomedicals, Solon, OH, USA). Then, the collected tissue was dissected into small pieces (<1 mm^2) and digested with 0.25% collagenase for 12 h. The liberated cells were collected and plated in 100 mm plastic tissue culture dishes with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotic–antimycotic solution, containing 100 U ml^{-1} Penicillin G sodium, 100 μg ml^{-1} Streptomycin sulfate and 250 ng ml^{-1} Amphotericin B in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. The cells were passed twice when they are at 80% confluency. After the second passage, the cells were detached using 0.25% Trypsin–1 mM EDTA-4Na and seeded directly onto either the cell culture-grade polystyrene dish, the untreated resin or NAC-added resin at a density of 2×10^4 cells cm^{-2}. The medium was renewed every 3 days. This study protocol was approved by the University of California at Los Angeles Chancellor's Animal Research Committee.

2.3. Detection of cell viability and apoptosis

The viability and apoptosis of the oral fibroblasts was evaluated by flow cytometric detection of annexin V binding and propidium Iodide staining (Annexin V-FITC Kit, BD Bioscience, San Jose, CA, USA). This method is based on the binding properties of annexin V to phosphatidylserine (PS) and on the DNA-intercalating capabilities of propidium iodide (PI). The cells, incubated for 24 h on untreated resin or NAC-added resin, were tested, and the intensity of PI staining (*y*-axis) was plotted versus FITC intensity (*x*-axis). In all four plots, viable cells were seen in the left lower quadrant (3: annexin V – negative/PI-negative), early apoptotic cells in the right lower quadrant (4: annexin V – positive/PI-negative), late apoptotic/necrotic cells in the right upper quadrant (2: annexin V – positive/PI-positive), and necrotic cells in the left upper quadrant (1: annexin V – negative/PI-positive) [22–24].

2.4. Evaluation of cell density

At culture days 2 and 5, the cells were gently rinsed twice with PBS and treated with 0.1% collagenase in 300 μl of

0.25% trypsin–1 mM EDTA-4Na for 5 min at 37 °C. A hematocytometer was used to count the number of detached cells. SEM was used for the selected culture to confirm the absence of any cell remnant on the substrates.

2.5. Gene expression analysis

The levels of gene expression were semiquantitatively analyzed using reverse transcription-polymerase chain reaction (RT-PCR). At day 7 of culture, total RNA in the cultures was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and purification column (RNeasy, Qiagen, Valencia, CA, USA). Following DNase I treatment, 1 μg of total RNA was reverse-transcripted into cDNA template using MMLV reverse transcriptase (Clontech, Carlsbad, CA, USA) and oligo(dT) primer (Clontech, Carlsbad, CA, USA) at 42 °C for 1 h and at 94 °C for 5 min. The PCR was performed using TaqDNA polymerase (Ex taq, Takara Bio, Madison, Wi, USA) to detect α-I type I collagen and α-I type III collagen mRNA. The forward and backward primers were designed; collagen I: 5′-GG CAACAGTCGATTCACC-3′ and 5′-AGGGCCAATGT CCATTCC-3′, collagen III: 5′-CCTGGA CCTCAGGGT ATC-3′ and 5′-TGCAGGGCC TGGACTACC-3′, respectively. The annealing temperature and the amplification cycles were set, respectively, 58 °C and 28 cycles for collagen I and 60 °C and 25 cycles for collagen III. The resulting products were visualized on a 1.5% agarose gel with ethidium bromide staining under UV light. The intensity of the bands was quantified using a densitometry analyzer (LAS-3000, FujiFilm, Tokyo, Japan) and normalized with reference to glyceraldeyhde-3-phosphate dehydrogenase (GAPDH-housekeeping gene) mRNA.

2.6. Collagen production

To quantify collagen production, Sirius red staining-based colorimetric assay was employed. Cultures from different groups were washed with pre-warmed 1× PBS at 37 °C for 1 min and fixed with Bouin's fluid for 1 h at room temperature. The cultures were washed with dd H_2O and treated with 0.2% aqueous phosphomolybdic acid (PMA) for 1 min. Then, the cultures were washed again with dd H_2O and stained with Sirius red dye (C.I. No. 35780, Pfaltz and Bauer, Stamford, CT, USA) dissolved in saturated aqueous picric acid (pH 2.0) at a concentration of 100 mg/100 ml for 90 min with mild shaking. The cultures were washed with 0.01 N hydrochloric acid for 2 min to remove all non-bound dye. Afterwards, 600 μl of 0.1 N sodium hydroxide was added to dissolve the staining using a microplate shaker for 30 min at room temperature. Then, the optical density (OD) of the solution was measured using a spectrophotometer at 550 nm against 0.1 N sodium hydroxide as a blank.

2.7. Three-point bending test

The mechanical properties of the untreated control bone cement and bone cement supplemented with 0.6% NAC were assessed from the transverse strength and elastic modulus obtained in a three-point bending test. The three-point bending test (transverse test) was performed with a rectangular specimen of bone cement at a cross-head speed of 1 mm min^{-1} using an Instron Testing Machine Autograph (AGF-500D, Shimadzu, Japan), at a controlled room temperature. Bone cement was mixed according to manufacturer's specifications, as described above, and placed into a preformed stainless steel rectangular mold (2×2×25 mm). After incubating the specimens in 37 °C for 24 h, the resin specimen was removed from the mold and polished with a #1000 abrasive sand paper. The dimensions of the specimen were measured. The transverse strength (D) and elastic modulus (E) were calculated by the equations $D = 3 \times Ll/2wt^2$ and $E = 3Sl/4 \times 3wt$, respectively, where L is the maximum load (L) exerted on the specimen, l is the distance between the supports (20 mm), w is the width, t is the depth of the specimen, and S is the slope of the initial load–displacement curve.

2.8. Statistical analysis

The numbers of samples was 3 for the cell density, flow cytometry, collagen production assays and RT-PCR. Further, a whole set of these experiments were performed 3–5 times, and reproducibility of the data was confirmed. The number of samples for the mechanical properties was 6. One-way ANOVA was employed to examine differences in cell density, flow cytometry results, collagen production and gene expression levels between the untreated and NAC-supplemented resin cultures. When needed, Bonferroni multiple comparison testing was used. The level of statistical difference was defined as $p < 0.05$. The t-test was used to examine differences in mechanical properties between the specimens with and without NAC.

3. Results

3.1. Higher viability of fibroblasts on NAC-containing resin

Flow cytometric analysis revealed that the percentage of viable cells 24 h after the incubation was remarkably lower in the culture on the resin substrate than that on polystyrene (Fig. 1A). However, cultures on resin substrates supplemented with NAC showed higher percentages than the culture on untreated resin. The percentages of viable fibroblasts were 28% and 35% on the resins with 0.4% and 0.6% NAC, respectively, while it was less than 2% on the untreated resin (Fig. 1A). More than 90% of the cells were viable in the culture on polystyrene. The percentages of necrotic cells and late apoptotic/necrotic cells were reduced NAC concentration-dependently.

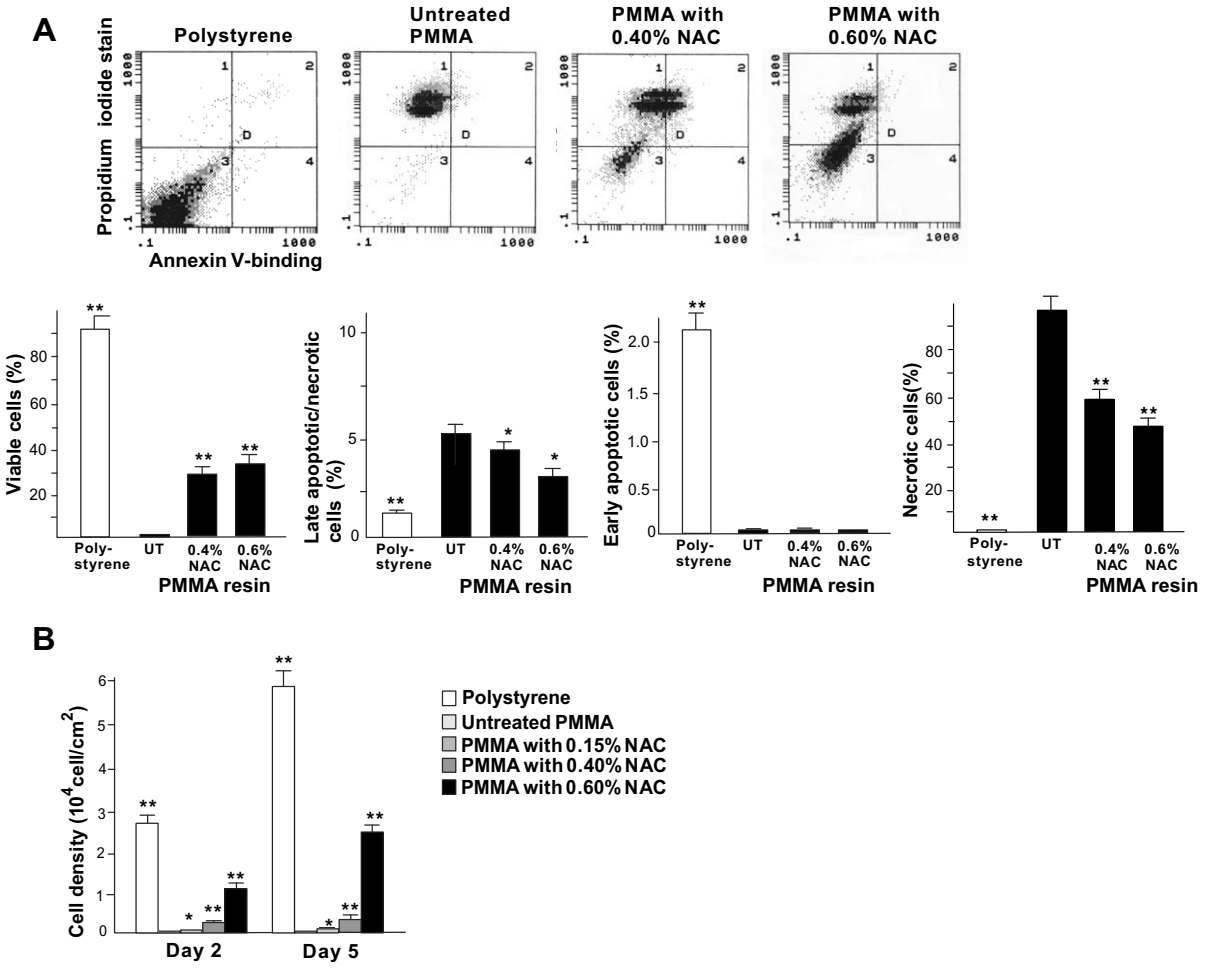

Fig. 1. (A) Results of the cell viability of oral fibroblasts 24 h after seeding onto the untreated dental resin, NAC-supplemented PMMA resins and polystyrene. The flow cytometric images are shown on the top, and the percentages of early apoptotic cells (quadrant 4 on the top images), late apoptotic/necrotic cells (quadrant 2), viable cells (quadrant 3) and necrotic cells (quadrant 1) are shown on the bottom. Ut: untreated control PMMA resin. Data are shown as the means ± SD ($N = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$ significant difference compared to the untreated (UT) PMMA resin. (B) Evaluation of cell density of oral fibroblasts cultured on untreated dental resin and NAC-supplemented PMMA resins. The results were obtained by counting cell number. Data are shown as the means ± SD ($N = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$ significant difference compared to the untreated PMMA resin.

3.2. Higher cell density on NAC-containing resin

Cell density was consistently higher on the NAC-supplemented resin compared to that on the untreated resin at days 2 and 5 (ANOVA, $p < 0.01$) (Fig. 1B). A positive correlation was found between the concentration of NAC and cell number, with 0.6% NAC-supplemented resin culture showing the highest cell number. Although the cell number was still lower than the culture on polystyrene, the culture on the 0.6% NAC-supplemented resin showed approximately 100 times greater number of the cells than the culture on the untreated resin at days 2 and 5 (Bonferroni, $p < 0.01$).

3.3. Restored fibroblastic gene expression on NAC-containing resin

Representative electrophoresis images and the standardized expression levels of collagen I and III from RT-PCR semiquantitative analysis are presented in Fig. 3 (top panels). The quantitative analyses revealed that the downregulated expression of both genes in the cells on the untreated resin was substantially recovered on the resin substrate supplemented with 0.60% NAC (histogram in Fig. 2). The cells on the 0.6% NAC-supplemented resin showed three times higher level of collagen I expression than those on the untreated resin. The level of collagen III expression was also 2.6 times higher on the 0.6% NAC-supplemented resin than on the untreated resin culture. Those expression levels, however, did not reach the level of the culture on polystyrene.

3.4. Higher collagen production on NAC-containing resin

Some areas of fibroblastic cultures were positively stained by Sirius red for the detection of collagen molecules (top panels in Fig. 3). At day 7 of culture, the amount of collagen was approximately three times greater for the cul-

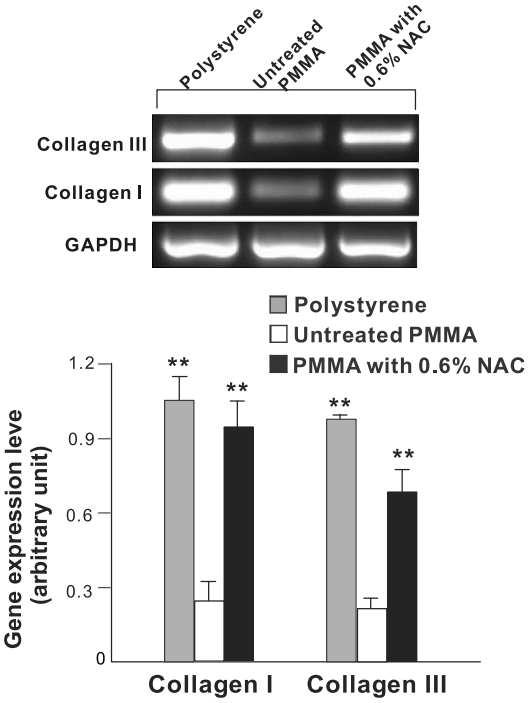

Fig. 2. Expression of collagen I and III genes analyzed by reverse transcriptase-polymerase chain reaction method (RT-PCR). The upper panel shows representative electrophoresis images visualized with ethidium bromide staining. The lower panel shows the intensity of band of the target genes normalized with reference to GAPDH expression level. Data are shown as the means ± SD ($N = 3$). $^{**}p < 0.01$ significant difference compared to the untreated PMMA resin.

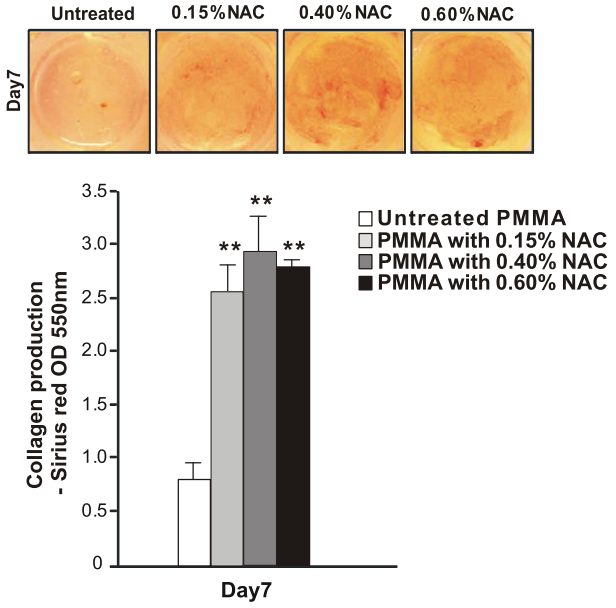

Fig. 3. Evaluation of collagen production by Sirius red staining-based colorimetric assay at day 7. The upper panel shows representative images of the cultures after the staining. Results of quantification of collagen deposition are shown in the lower panel. Data are shown as means ± S.D ($N = 3$). $^{**}p < 0.01$ significant difference compared to the untreated PMMA resin.

tures on NAC-supplemented resin than the culture on the untreated resin (histogram in Fig. 3). No significant difference was found among the cultures with different NAC concentrations ($p > 0.05$, Bonferroni) (Fig. 3).

3.5. Mechanical properties of PMMA resin not affected by NAC incorporation

There were no differences in transverse strength and elastic modulus obtained from three-point bending tests between the resin specimens with and without 0.6% NAC incorporation (Fig. 4).

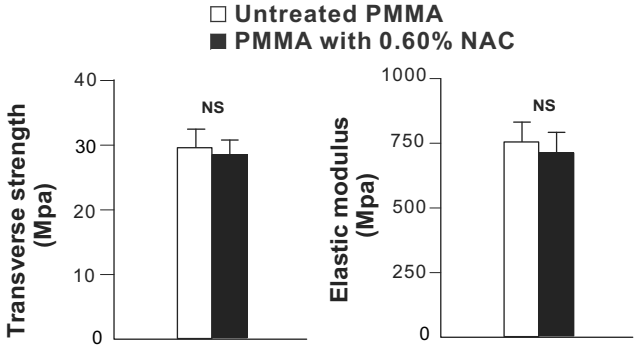

Fig. 4. Mechanical properties unaffected by NAC supplementation. Transverse strength (a) and elastic modulus (b) of the resin specimens with and without 0.6% NAC incorporation. Data are shown as the means ± SD ($n = 6$). NS: not significant between the specimens with and without NAC.

4. Discussion

Cytotoxicity is a primary factor of biocompatibility and is generally determined by in vitro cell culture. The exposure of fibroblasts to PMMA resin material in this study resulted in an overwhelming cell death and significant suppression of fibroblastic function, characterized by suppressed proliferative activity, downregulated expression of fibroblastic genes and reduced collagen production. Several studies reported that PMMA resin materials and their eluates have cytotoxic effects on human oral fibroblasts [25,26] and lead to inhibition of cell growth, DNA replication, RNA synthesis and metabolic processes [27–29].

In this study, the percentage of viable cells was only 2% on the untreated resin culture. Generally, there are two modes of cell death, i.e. apoptosis and necrosis [30]. Apoptosis is a programmed physiological process of cell death triggered by specific stimuli through specific transcriptional pathways. It plays a critical role not only in normal development, but also in the pathology of a variety of diseases and the activity of a large number of toxicants. The mechanisms leading to apoptosis have been extensively reviewed [31]. In contrast to apoptosis, necrosis generally sets off a tissue inflammation process associated with clinical symptoms [31]. It is usually caused by unique events, such as degradation of chromatin and cytoskeletal and

membranous breakdowns. The role of PMMA resin in triggering cell apoptosis and necrosis has been demonstrated previously. In an in vitro study, most oral fibroblasts cultured on PMMA resin died by means of necrosis rather than apoptosis [26]. In another study, eluates from denture base PMMA resin enhanced cell death via necrosis [32]. In this study, the majority of cells died via necrosis. However, it should be noted that different cell culture conditions and resin components, incubation times and dosages may result in different proportions of apoptotic and necrotic cells between different studies. In this study, we cultured fibroblasts directly on the resin. We designed this experiment simulating intraoral procedures of denture reline or extension following tooth extraction, where oral fibroblasts are in contact with newly added self-curing resin or exposed to the monomer components released from the polymerizing resin. This experimental design may explain the high percentage of cell death in this study (percentage of viable cell was 2%). More importantly, it should be noted that, despite this most detrimental condition, NAC was effective to improve the cell viability and restore the cellular function.

Although not clear yet, there are two known mechanisms underlying the adverse effects of resin materials; genetic damage and an oxidative stress causing from an imbalance between reactive oxygen species (ROS) and anti-oxidant redox defensive system. Monomers released from resin materials above a certain concentration cause DNA damage that results in a delay or arrest of a cell cycle [4,33,34]. Resin monomers increase intracellular ROS, as represented by hydrogen peroxide, superoxide anions, and hydroxyl radicals, and subsequently reduce the intercellular level of antioxidant molecules like glutathione (GSH), a direct ROS scavenger [16,35,36]. The increased ROS after the GSH depletion may induce cytotoxicity by modulating the signaling pathways leading to cell death [37,38]. In addition, ROS may directly damage the cellular structure. In this study, the fibroblastic function, as demonstrated by gene expression and collagen production, was substantially diminished when fibroblasts were cultured on the untreated PMMA resin, indicating that the MMA monomer concentration produced by its initial and continuous release from the PMMA resin was high enough to generate cytotoxicity.

The addition of NAC to the resin improved the percentage of the viable cells up to 35% compared to less than 2% without NAC. The improvement was mostly due to the reduced number of necrotic cells and late apoptotic/necrotic cells. However, even with 0.6% NAC incorporated in the resin, flow cytometric analysis showed the 2-phasic distribution of cell population; a population in the viable cell zone and other in the necrotic and late apoptotic/necrotic zone; not all of the cells were rescued by NAC. There are two possible pathways of NAC-mediated protection of the cells: (1) NAC-mediated detoxification of resin material by in situ scavenging of free radicals; (2) NAC-mediated restoration of cellular antioxidant system by supplying antioxidants. If the material per se had been detoxified, all of the cells should have been rescued. However, only 35% of the cells were viable on the resin containing 0.6% NAC. Although hypothetical, NAC released from the resin may have served to supply the cells with antioxidant molecules as a GSH precursor or direct radicals scavenger outside the resin, and NAC at this concentration was not sufficient in dose to rescue all of the cells. Since the percentage of viable cells and cell density continued to increase with an increase of NAC concentration, testing higher concentrations of NAC would be necessary for further improvement of the percentage of viable cells.

The rescued cells were able to manifest proliferative activity, synthesize collagen I and III mRNA and deposit collagen. Therefore, these results indicated that, although the viability rate did not reach that found in the polystyrene culture without resin, that NAC remarkably ameliorated the cytotoxicity of the resin to a biologically significant degree. Among the NAC-supplemented resin cultures, the 0.6% NAC-added resin culture demonstrated the highest cell number. For this reason, this group was chosen for evaluating the expression level of fibroblastic genes. For both genes tested in this study, the downregulated expression by PMMA resin was restored to nearly a similar level of the untreated control by the incorporation of NAC, indicating that fibroblasts can be maintained in the normal lineage of activity under the co-existence of NAC and the resin.

As for collagen colorimetry, the results showed that NAC addition to the resin significantly increased collagen deposition in all groups. This means that NAC was able to restore the function of fibroblasts in secreting collagenous molecules into the extracellular matrix. The employed Sirius red stain assay specifically detects collagen type I and III molecules [39]. Such an increase in collagen deposition in NAC-added resin groups can be attributed to the higher number of viable cells and restored activity of fibroblasts compared to untreated resin group. The results of this study clearly demonstrate that NAC has succeeded to ameliorate the cytotoxicity and restore the cellular function of fibroblasts. This was accomplished in a concentration-dependent manner. The exact mechanism by which NAC detoxifies PMMA and rescue cells is not fully understood. It is suggested that the addition of NAC molecules may have contributed directly in removing ROS and indirectly in compensating the depletion of GSH; helping to improve the disrupted ROS removal redox system [4].

The rescue from cell death and restoration of impaired function by incorporation of NAC in the self-curing resin was also reported in dental pulp cells [21]. The incorporation of 0.6% NAC increased the percentage of viable cells from 10% to 70% of the cells. Having known that the change of cell viability was from 2% to 35% in the present results using oral fibroblasts, fibroblasts may be more susceptible to the toxicity of this type of materials. However, the improvement was even higher for the fibroblasts, indicating that the NAC is, at least, equivalently effective to

both cell types. As for the function of dental pulp cells, alkaline phosphatase activity of the cells continued to increase even with 0.6% NAC incorporation, although the fibroblasts used in this study showed the saturated improvement of collagen synthesis between 0.15% and 0.6% NAC. The sensitivity to NAC, in terms of the restoration of cellular function, may differ among different cell types. A recent study also showed the effective NAC-mediated detoxification of composite resin to dental pulp cells [40], suggesting the effectiveness of NAC to various polymerizing materials. Also, the study demonstrated the in situ protection of dental pulp cells in the rat incisors and in vitro protection of human primary dental pulp cells, suggesting a necessity to explore these findings toward the clinical application.

Although the present results from RT-PCR showed that an addition of 0.6% NAC restored the gene expression to the level closer to that on polystyrene culture, it may be immature to conclude that this is the maximum to improve biocompatibility of the material. The cell density and percentage of viable cells did not seem to reach a plateau of their improvement. Further studies will be needed to determine the optimum concentration of NAC. Another important issue that needs attention is the physical and mechanical properties of NAC-supplemented resin. Mechanical strength and elastic modulus of the resin specimens were not influenced by the incorporation of NAC at the highest concentration we tested, indicating no significant adverse effect of NAC on polymerization behavior within the limitation of this study. It is necessary to determine whether NAC addition affects other physical and mechanical properties of PMMA resin, such as hardness, mechanical fatigue, polymerization time, surface morphology and color change and stability in future studies. Such an evaluation would provide guidelines to design NAC-added resin that has the most favorable biological effect without compromising other necessary properties.

In conclusion, an auto-polymerizing PMMA dental resin material demonstrated high cytotoxicity that resulted in devastating cell death and suppressed function of oral fibroblasts. The incorporation of NAC in the resin material ameliorated the cytotoxicity to a biologically significant degree, as represented by the restored gene expression and collagen producing capability, without adversely affecting strength and elastic modulus of the material. This NAC-assisted prevention of cellular toxicity may provide new insights into the recognition of risk factors and candidate solutions for the future development of PMMA-based resin materials.

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