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Restored viability and function of dental pulp cells on poly-methylmethacrylate (PMMA)-based dental resin supplemented with N-acetyl cysteine (NAC)

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ABSTRACT

This study examines cytotoxicity of poly-methylmethacrylate (PMMA)-based dental temporary filling resin to dental pulp cells, and the potential amelioration of the toxicity with an anti-oxidant amino-acid, *N*-acetyl cysteine (NAC). Dental pulp cells extracted from rat maxillary incisors were cultured on the resin material with or without NAC incorporation, or on the polystyrene. The cultures were supplied with osteoblastic media, containing dexamethasone. Forty five percent of cells on the PMMA dental resin were necrotic at 24 h after seeding. However, this percentage was reduced to 27% by incorporating NAC in the resin, which was the level equivalent to that in the culture on polystyrene. The culture on the untreated resin was found to be negative for alkaline phosphate (ALP) activity at days 5 and 10 or von Kossa mineralized nodule formation at day 20. In contrast, some areas of the cultures on NAC-incorporated resin substrates were ALP and von Kossa positive. Collagen I and dentin sialoprotein genes were barely expressed in day 7 culture on the untreated resin. However, those genes were expressed in the culture on the resin with NAC. These results suggest that the decreased cell viability and the nearly completely suppressed odontoblast-like cell phenotype of dental pulp cells cultured on PMMA dental resin can be salvaged to a biologically significant degree by the incorporation of NAC in the resin.

1. Introduction

Acrylic-based self-polymerizing resin, consisting of a solid part of a prepolymerized poly-methyl methacrylate (PMMA) and a liquid part of methyl methacrylate (MMA), is one of the most frequently and extensively used materials in daily dental practice. Most common use of the material includes the fabrication of temporary crowns and the temporal seal of inlay cavities, where the PMMA resin is directly loaded onto prepared teeth. Under these circumstances, the direct chemical and biological effects of such materials on dental pulp cells/tissue via the exposed dental tubules are unavoidable to some degree [1–5].

A number of in vitro and in vivo studies revealed the toxicity and adverse effects of MMA-based resin at the cellular and tissue levels. The resin extract inhibits osteoblastic cell proliferation [6]. The monomer released from the resin induces apoptosis and secondary necrosis of osteoblastic cell lines [7]. The monomer effects on gene mutation resulting in delayed cell cycle and cell death have been extensively demonstrated

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in fibroblasts and fibroblastic cells [8,9]. Necrosis, fibrosis and histocytosis are locally found in the tissue around resin materials [10,11]. The mechanisms of these detrimental and, often times critical, effects involve direct toxicity of released or residual MMA monomer and oxidative stress created by free radicals during the polymerization process [12,13]. Despite these evidences of MMA toxic effects on cell viability and proliferative activity, the MMA effects on cellular function and phenotype have rarely been investigated. More importantly, the MMA effect on dental pulp cells, in spite of its great clinical relevance, is rarely studied.

N-acetyl cysteine (NAC) is known as an anti-oxidant cysteine derivative and metabolized into glutathione [14] that serves as an important non-enzymatic factor in the intracellular anti-oxidant system [12,15]. NAC also acts as a direct, strong oxidant scavenger [16]. In fact, the addition of NAC to the culture inhibited triethylene glycol dimethacrylate (TEGDMA)- and 2-hydroxyethyl methacrylate (HEMA)-induced cell death and cell cycle arrest in fibroblasts [12,17–19].

We hypothesized that the PMMA-based dental resin material is cytotoxic enough to critically suppress phenotypes and function of the dental pulp cells and that incorporation of NAC in the resin can ameliorate the resin-induced adverse effects. The objective of this study was to examine the effect of PMMA auto-polymerizing, temporary dental filling resin on cell viability and odontoblastic phenotypes of dental pulp cells and to determine whether the incorporation of NAC in the resin restores the possibly impaired odontoblastic function.

2. Materials and methods

2.1. *Resin preparation*

Untreated control PMMA dental resin was prepared by mixing the powder and liquid for 30 s according to the manufacturer's instructions (powder/liquid ratio of 0.6 g/0.4 g) (DuraSeal, Reliance Dental, Worth, IL, USA) in a 12-well culture plate (Fisher Scientific, Pittsburgh, PA, USA). The experimental NAC-incorporated resin was prepared by mixing the powder and liquid containing various concentration of NAC (0.15%, 0.4% or 0.6% in weight percentage of the final resin substrate). The mixed resin was incubated at 37 °C for 30 min and rinsed with ddH_2O once. This particular commercial PMMA product is used for temporary filling and seal of the prepared natural teeth such as inlay cavities and crown abutments, using the direct intra-oral application.

2.2. *Cell culture*

Dental pulp tissue was extracted from the upper central incisors of 8-week-old male Sprague–Dawley rats and treated with 0.1% collagenase (Sigma, St. Louis, MO, USA) in 0.25% Trypsin–1 mM EDTA–4Na (Gibco BRL Div. of Invitrogen, Gaithersburg, MD, USA) in 37 °C for 15 min. The pellet of released cells centrifuged at 10,000 rpm for 5 min was resuspended in alpha-modified Eagle's medium (Gibco BRL Div. of Invitrogen, Gaithersburg, MD, USA) supplemented with 15% fetal bovine serum (Gibco BRL Div. of Invitrogen, Gaithersburg, MD, USA), 50 mg/mL ascorbic acid (Sigma, St. Louis, MO, USA), 10 mM Na-β-glycerophosphate (Sigma, St. Louis, MO, USA), 10^{-8} M dexamethasone (Sigma, St. Louis, MO, USA) and antibiotic–antimycotic mixture solution supplemented with 10,000 units/mL Penicillin G sodium, 10,000 mg/mL Streptomycin sulfate and 25 mg/mL Amphotericin B antibiotics (Gibco BRL Div. of Invitrogen, Gaithersburg, MD, USA). The cells were incubated in 100-mm diameter culture dishes in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. At 80% confluency, the cells were detached using 0.25% Trypsin–1 mM EDTA–4Na and seeded onto either a cell culture-grade polystyrene dish (Fisher Scientific, Pittsburgh, PA, USA), untreated resin, or NAC-incorporated resin at a density of 5×10^4 cells/cm^2. The culture 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. *Flowcytometry*

An early sign of apoptosis in the dental pulp cells was evaluated by annexin-based flowcytometry (Annexin V-FITC Kit, BD Bioscience, San Jose, CA, USA). The method is based on the binding properties of annexin V (AV) to phosphatidylserine and on the DNA-intercalating capabilities of propidium iodide (PI). The cells, incubated on untreated resin, the NAC-incorporated resin or the culture-grade polystyrene dish for 24 h, were tested.

2.4. *Cell density measurement*

At 3 days after seeding, the attached cells were gently rinsed twice with PBS and detached with 0.1% collagenase in 300 □L of 0.25% trypsin, 1 mM EDTA–4Na for 15 min at 37 °C. A hematocytometer (Bright-Line, Hausser Scientific, Horsham, PA, USA) was used to count the number of the collected cells. The substrates were examined under scanning electron microscopy to confirm there were no remaining cells. Three independent cultures were prepared for the resin substrate with or without NAC ($n = 3$).

2.5. *Intra-cellular glutathione and superoxide dismutase (SOD) activity assays*

Oxidative stress generated in the culture by the PMMA resin substrate was evaluated by quantifying the amounts of glutathione as the most abundant non-enzymatic anti-oxidant and the activity of superoxide dismutase as a major anti-oxidative enzyme.

The total glutathione concentration in the culture was quantified by a fluorescent reagent kit (Total Glutathione Quantification Kit, Dojindo Molecular Technologies, Gaithersburg, MD, USA). The assay principal is that glutathione and 5,5′-2-nitrobenzoic acid (DTNB) react to generate glutathione disulfide (GSSG) and 2-nitro-5-thiobenzoic acid which has a yellow color, in parallel with the glutathione recycling by glutathione reductase.

To measure SOD activity, the colorimetric method was applied (Superoxide Dismutase Assay, Cell Technology, Mountain View, CA, USA). This method is based on the inhibition rate of SOD on the proportional reaction to produce formazan dye between WST-1, a highly water-soluble tetrazolium salt, and superoxide anions repeatly produced by the xanthine oxidase. These two dyes were respectively identified as absorbencies. Three independent cultures that were evaluated in each of the experimental groups ($n = 3$).

2.6. Alkaline phosphatase (ALP) activity

The day 5 and 10 cultured dental pulp cells were washed with Hank's solution twice, and incubated with 120 mM Tris buffer (pH 8.4) (Sigma, St. Louis, MO, USA) containing 0.9 mM naphthol AS-MX phosphate (Sigma, St. Louis, MO, USA) and 1.8 mM fast red TR (Sigma, St. Louis, MO, USA) for 30 min at 37 °C. The stained images were analyzed for ALP-positive area defined as [(stained area/total dish area) × 100)] (%) using a digitized image analysis system (ImageJ, NIH, Bethesda, MD, USA). Three independent cultures were evaluated in each the experimental groups ($n = 3$).

2.7. Gene expression analysis

The expression of odontoblastic related genes was analyzed using reverse transcription-polymerase chain reaction (RT-PCR). At culture day 7, 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, reverse transcription of 0.5 μg of total RNA was performed using MMLV reverse transcriptase (Clontech, Carlsbad, CA, USA) in the presence of oligo(dT) primer (Clontech, Carlsbad, CA, USA). The PCR reaction was performed using Taq DNA polymerase (EX Taq, Takara Bio, Madison, WI, USA) to detect alpha-I type I collagen (collagen I) and dentin sialoprotein (DSP) mRNA. The forward and backward primers (Operon Biotechnologies, Huntsville, AL, USA) were designed; collagen I: 5′-GGCAACAGTCGATTCACC-3′ and 5′-AGGGCCAATGTCCATTCC-3′, DSP: 5′-CGAGTCGATAGCCG-TAGGAG-3′ and 5′-AACTCCACTCCCGTGTGTTC-3′, respectively. Preliminary PCR trials were performed to determine the annealing temperature and the optimal number of cycles that yields the linear range of PCR amplification for each primer set. The conditions were described elsewhere [20]. The PCR amplification was performed at least three times with the determined condition to verify the consistency. Resulting products were visualized on 1.5% agarose gel (Invitrogen, Carlsbad, CA, USA) with ethidium bromide (Sigma, St. Louis, MO, USA). Band intensity was quantified under UV light, and the values were normalized with reference to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The isolated RNAs from 3 wells in each group were pooled into one tube and counted as one independent sample in each group ($n = 1$).

2.8. Von Kossa stain

To determine the mineralizing capability of the dental pulp cells on the temporary filling resins of various conditions, von Kossa staining was utilized. At day 20 of culture, the cells were fixed with a 50% ethanol/18% formaldehyde solution (Sigma, St. Louis, MO, USA) for 30 min. The cultures were then incubated with 5% silver nitrate (Sigma, St. Louis, MO, USA) under UV light for 30 min. Finally, the cultures were washed with ddH_2O twice and incubated with a 5% sodium thiosulfate solution (Sigma, St. Louis, MO, USA) for 2–5 min. The mineralized nodule area defined as [(stained area/total dish area) × 100)] (%) was measured using a digitized image analysis system. Three independent cultures were evaluated in each of the experimental groups.

2.9. Statistical analysis

One-way ANOVA was employed to examine differences in cell density, intra-cellular glutathione level, SOD activity and von Kossa-positive area among the experimental groups. Bonferroni multiple comparison was used as a post hoc test. Two-way ANOVA was applied to evaluate the effects of the culture condition and time point on ALP activity, followed by a post hoc test as needed. The level of statistical significance was defined as $p < 0.05$.

3. Results

3.1. Resin-induced cell death of dental pulp cells

The untreated resin caused necrosis in over 45% of the dental pulp cells; this was improved by the incorporation of NAC into the resin up to the level equivalent to that on the polystyrene (27%) (Fig. 1a). It was also a trend that the percentage of secondary necrotic cells was reduced by the NAC (Fig. 1a). Unlike necrosis, the change in the percentage of apoptotic cells was relatively small (Fig. 1a).

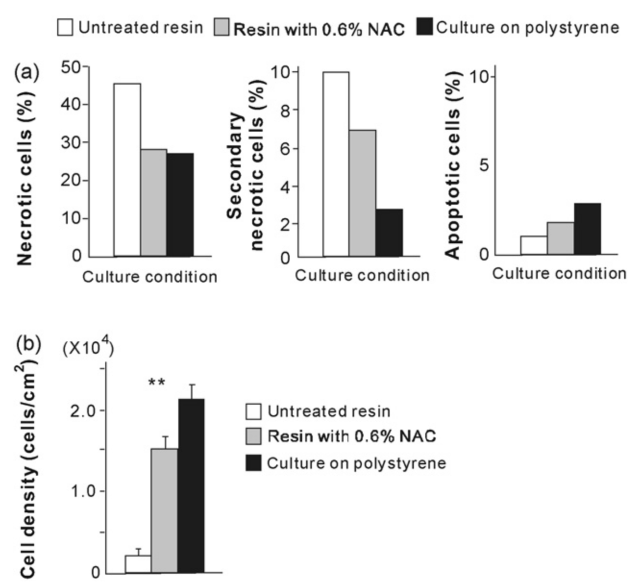

Fig. 1 – (a) Results of the annexin-based flowcytometry in dental pulp cell culture at 24 h after seeding onto the PMMA dental resin without NAC (untreated resin), the NAC-incorporated resin or the culture on polystyrene. The percentages of necrotic cells [annexin V (−)/propidium iodide (+) cells], secondary necrotic cells [annexin V (+)/propidium iodide (+) cells] and apoptotic cells [annexin V (+)/propidium iodide (−) cells] are shown. (b) Results of cell density shown as means ± S.D. ($n = 3$). **Statistically significant (One-way ANOVA, $p < 0.01$).

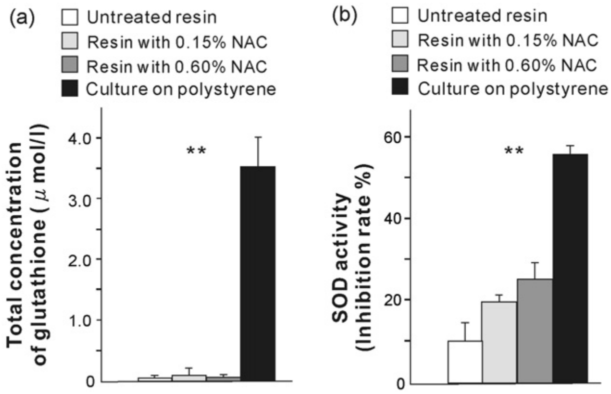

Fig. 2 – (a) The total concentration of glutathione and (b) superoxide dismutase (SOD) activity of the dental pulp cells at 24 h after seeding. Data are shown as means ± S.D. ($n = 3$). **Statistically significant difference among the groups (One-way ANOVA, $p < 0.01$).

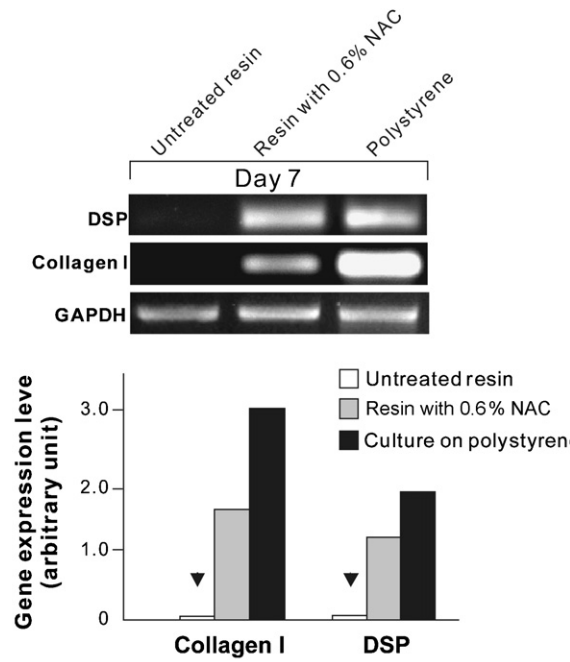

Fig. 3 – Expressions of collagen I and dentin sialoprotein (DSP) genes analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) at day 7. The upper panel shows representative electrophoresis image of collagen I and DSP gene expression visualized with ethidium bromide staining. The lower panel shows the band intensity of collagen I and DSP normalized with reference to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression level ($n = 1$). Note that these odontoblast-like gene expressions are almost completely suppressed in the culture on the untreated resin (arrows).

One-way ANOVA showed that there was significant difference in cell density at day 3 among the experimental groups ($p < 0.001$) (Fig. 1b). The cell density on the untreated control resin was less than 10% of that on the polystyrene (Bonferroni comparison, $p < 0.01$), which was increased 7 times by the incorporation of NAC in the resin.

3.2. *Intra-cellular glutathione level and SOD activity*

There was a statistically significant difference in the intra-cellular glutathione level among the experimental groups (One-way ANOVA, $p < 0.01$) (Fig. 2a). Intra-cellular glutathione was reduced remarkably in the dental pulp cells cultured on the resin substrates with or without NAC compared to the culture on polystyrene (Bonferroni, $p < 0.01$). There was a significant difference in SOD activity among the groups (One-way ANOVA, $p < 0.01$) (Fig. 2b). The SOD activity was also reduced in the untreated resin culture. However, it was recovered on the NAC-incorporated resin in a concentration-dependent manner (Bonferroni comparison, $p < 0.01$).

3.3. *Odontoblast-like function of dental pulp cells*

Representative electrophoresis images and the standardized expression level of collagen I and dentin sialoprotein genes at day 7 are presented in Fig. 3. The expression of collagen I was barely detected in the culture on the untreated resin, while it was detected in the culture on the resin with 0.6% NAC. The level of collagen I expression was recovered by NAC to the level which was 50% of the culture on the polystyrene. Likewise, DSP gene was hardly expressed in the untreated resin culture. The incorporation of NAC into the resin restored the expression, even though the level of the expression was still lower than that in the culture on the polystyrene.

The dental pulp cells cultured on the untreated resin showed nearly no ALP-positive stain at days 5 and 10 (Fig. 4). In contrast, some areas of the NAC resin cultures were found to be ALP positive. Two-way ANOVA showed that there was no significant interaction between the culture time and experimental groups ($p > 0.05$). One-way ANOVA applied to each of the culture days of 5 and 10 showed that ALP-positive area differed significantly among the different culture conditions. The cultures on the NAC-incorporated resin showed a greater ALP-positive area than the culture on the untreated resin at both culture days (Bonferroni, $p < 0.01$), although they were lower than that of the culture on the polystyrene ($p < 0.01$). The increase of ALP-positive area on the resin substrates was NAC dose-dependent until 0.4% NAC; the incorporation of 0.6% NAC in the resin did not result in further gain of the ALP detection at both days 5 and 10.

There was significant difference in Von Kossa-positive area among the groups (One-way ANOVA, $p < 0.01$) (Fig. 5). Like ALP activity, the culture on the untreated resin showed nearly no Von Kossa-positive area at day 20, whereas the culture on the NAC-incorporated resin exhibited some von Kossa-positive areas (Bonferroni comparison, $p < 0.01$) (Fig. 5). The von Kossa-positive area increased on the NAC-incorporated resin substrates in a NAC concentration-dependent manner up to 0.40%, followed by a saturated trend. Over 15% of von Kossa-positive area was obtained on the 0.4% and 0.6% NAC-incorporated resin, which was approximately 50% of the level in the culture on the polystyrene.

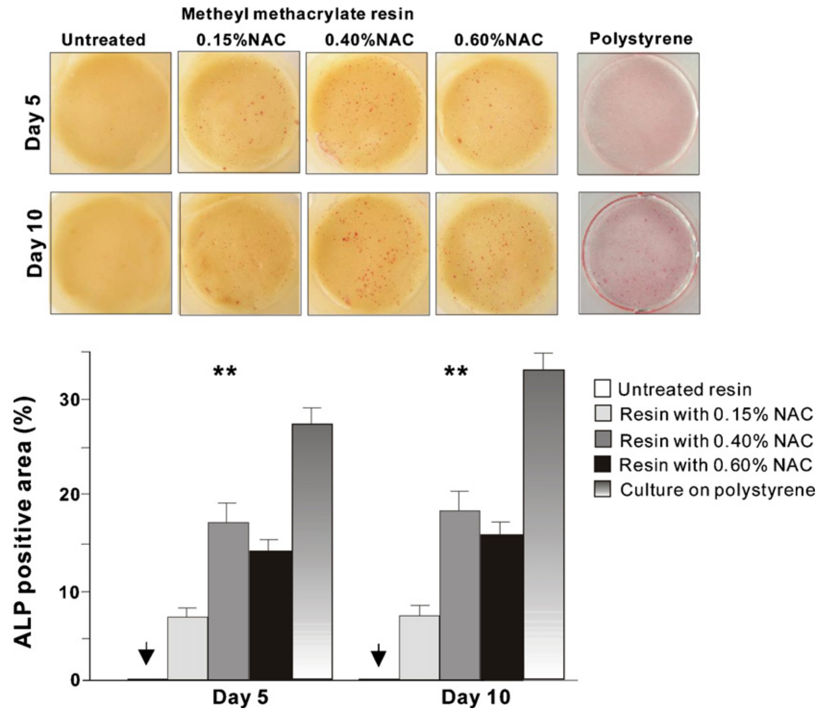

Fig. 4 – Alkaline phosphatase activity of the dental pulp cells at day 5 and day 10. Representative images of ALP staining are shown on the top. The percentage of the ALP-positive area relative to the culture area are shown on the bottom as the mean ± S.D. ($n = 3$). Note that ALP activity is almost completely suppressed in the culture on the untreated resin (arrows). **Statistically different among the groups ($p < 0.01$) (One-way ANOVA).

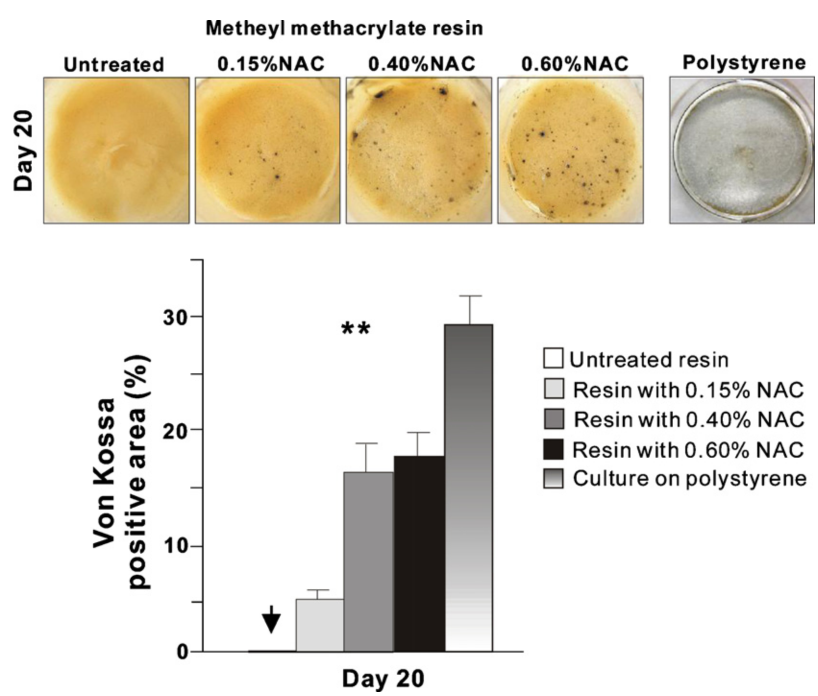

Fig. 5 – The matrix mineralizing capability of dental pulp cells at day 20, evaluated by von Kossa staining. Representative images of von Kossa stain are shown on the top. The percentage of the von Kossa-positive area relative to the culture area is shown on the bottom as the mean ± S.D. ($n = 3$). Note that von Kossa-positive mineral deposition is almost completely suppressed in the culture on the untreated resin (arrows). **Statistically significant difference among the groups ($p < 0.01$) (One-way ANOVA).

4. Discussion

This study showed that culturing dental pulp cells on PMMA dental resin material caused their cell death to a critical degree and the nearly complete inhibition of odontoblastic phenotype and function. More importantly, the incorporation of NAC into the resin salvaged the dental pulp cells from resin-induced death and substantially restored their phenotype and function.

To confirm the NAC-ameliorated inhibition of function in dental pulp cells, we examined several phenotypical and differentiation markers. The early stage markers of the odontoblastic phenotype of the dental pulp cells, such as ALP activity and collagen I gene, and such late stage markers as DSP gene expression and matrix mineralization were expressed in the culture on the NAC-incorporated resin, while these phenotypes were almost completely suppressed on the untreated resin substrate. These markers are typically seen as odontoblast-like cell phenotypes in the dental pulp cells cultured in the osteoblastic media [20]. It has been, therefore, demonstrated that NAC is effective in helping to restore odontoblastic differentiation from its initial to mature stages.

There are two mechanisms underlying the adverse effects of the resin monomer on the tissue and/or cells [12]. One is the genetic damage caused by ROS arisen from resin components, such as hydrogen peroxide, superoxide anion and hydroxyl radical, and resin monomer per se [21–26]. High concentrations of resin monomer, such as TEGDMA or HEMA induce the formation of micronuclei, indicating chromosomal aberrations [23], and the initiation of DNA strand breaks [21,22]. Molecular analyses have also shown that TEGDMA induces a characteristic spectrum of mutations in the hprt gene in V79 cell line, resulting in the total deletions of the entire exon sequence of this genetic locus [26]. Moreover, these monomers when exceeding some level may interfere with the cell cycle due to confusing G1/S and G2/M check points through p53-dependent or independent pathways, which leads to irreversible cell damage or programmed cell death [12,24,25].

The other mechanism responsible for the monomer toxic effect is the oxidative stress occurring from an imbalance between ROS (reactive oxygen species) and the anti-oxidant redox system [8,12,27–29]. ROS is thought to reduce the intercellular level of anti-oxidant molecules by depleting the constituent anti-oxidants, as represented by glutathione, a non-enzymatic direct ROS scavenger [19,30,31]. Such excessive ROS causes the biologically adverse effects not only by directly injuring the cellular components [28], but also by indirectly regulating the signaling pathways inducing cell death [8,27,29]. However, the present result showed no change of the percentage of apoptotic cells between any substrates including the polystyrene. Instead, the necrotic cells were substantially decreased after NAC incorporation into the resin. It is known that TEGDMA induces cell death via apoptosis by inhibiting the phosphorylation of protein kinase B (PKB)/Akt [29] which plays a central role in signaling as a downstream target for the phosphatidylinositol 3-kinase (PI3-K) mediated cell survival pathway [32]. The toxicity of the PMMA resin used in this study may have been sufficiently high to induce direct structural damages in the cell, instead of triggering the apoptotic signals.

NAC is a cysteine derivative and has sulfhydryl groups that are positively charged. It can be intra-cellularly metabolized to L-cyctein, a glutathione precursor [14,33]. NAC functions as a direct anti-oxidant scavenger of ROS due to electrical neutralization between sulfhydryl groups and ROS [16] and as a resource of glutathione to compensate the intra-cellular glutathione level depleted by ROS, which help maintain the balance of the anti-oxidant redox system [33]. NAC appears to decrease the oxidative stress and reduce biologically negative impacts such as apoptosis, micronuclei, DNA base sequence defects and cell cycle arrest initiated by resin monomer in fibroblasts [13,17–19,34,35]. Loss of SOD activity is one of the representative characteristics of cells under oxidative stress [36]. In fact, SOD activities in the dental pulp cells were remarkably reduced by the resin and recovered by NAC in a dose-dependent manner. In contrast, NAC did not have an effect on the intra-cellular glutathione level, which was probably due to the nearly complete depletion of glutathione by the resin. Further interest of this research should include the investigation of higher concentrations of NAC to prevent the depletion of GSH.

Although the role of NAC on various redox-sensitive systems is complex and far from being fully understood, it has been hypothesized that NAC may act on the sulfydryl groups of redox-sensitive intra-cellular signaling pathways. The pathways involve some transcription factors such as activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB), through which ROS may activate apoptosis-inducing signaling pathways [37–43]. Interestingly, these anti-oxidant-responsive proteins are thought to play a key role in regulating cellular differentiation [44,45]. For instance, AP-1 functions in the mitogen-activated protein kinase (MAPK) signaling pathway on osteoblastic and odontoblastic differentiation. [45–47] Further, AP-1 and NF-κB are considered to contain reactive cysteine residues that participate in a thiol-disulfide reaction through a redox status [12]. The interaction of NAC with these molecules will be of great interest to study.

It is important to design a cell culture study that resembles clinical situations. The PMMA resin product used in this study is used for the direct application to the prepared teeth. It is known that when loaded onto prepared teeth, resin monomers including HEMA and TEGDMA start to diffuse through dentin in sufficient concentrations to exert their biological adverse effects on the dental pulp cells [12,19]. Therefore, culturing the dental pulp cells directly on the resin substrate was considered as an experimental set-up in this study.

The commercial PMMA-based dental resin products are frequently used in the daily dental practice and have achieved successful outcomes as clinically essential materials due to their many advantages. Despite the efforts to improve the mechanical properties and curing behaviors of PMMA resin, its biological effects seem to have been underestimated. Auto-polymerized PMMA dental resin is directly applied to prepared teeth, and it consistently releases its components, such as monomer, from the initial application phase through post-polymerization [48]. In particular, temporary filling resin materials such as these used in this study are designed to polymerize incompletely and are likely to release more monomer [49]. This present study demonstrated the crucial adverse effects of temporary filling resin, as well as the successful effects of NAC in ameliorating these effects. The toxicity-control approach using anti-oxidant agents, such as NAC, should be considered in the future to develop more bio-friendly resin materials.

5. Conclusion

Culturing rat dental pulp cells on the PMMA-based temporary filling dental resin resulted in the critical levels of cell death and functional suppression. The incorporation of NAC in the resin ameliorated these adverse effects to a biologically significant degree.

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