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N-acetyl cysteine erases cellular damage and oxidative stress by PMMA on oral epithelial cells

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Abstract

Introduction: Polymethylmethacrylate (PMMA) resin has a concern about induction of irritant contact inflammation or allergic reaction on oral mucosa in association with oxidative stress. N-acetyl cysteine (NAC), an antioxidant amino acid derivative, alleviates cytotoxicity of resin materials. The purpose of this study was to investigate if incorporation of NAC into self-curing PMMA resin eliminates cellular damage and oxidative stress of the extracts on cultured oral mucosal epithelial cells.

Material and methods: A rat or human oral mucosal epithelial confluent monolayer culture on polystyrene was incubated with Dulbecco’s modified Eagle’s medium–Nutrient Mixture F-12 or oral keratinocyte medium with or without extracts from commercial self-curing PMMA-based dental resin with or without pre-incorporation of NAC. Cell structure damage and invasion of foreign matter into cells were evaluated on day 1 in rat cell cultures by quantification of attached cell number, lactate dehydrogenase (LDH) release, amount of E-cadherin, intracellular formaldehyde and lysosomal levels. Status of oxidative stress and inflammation were evaluated on day 1 in human cell culture by quantification of levels of intracellular total and oxidized glutathione and reactive oxygen species (ROS) and multiplex immunoassay for proinflammatory cytokine release. Formaldehyde content and 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capability in the extracts were measured to evaluate their degree of substance elution and antioxidant ability.

Results: Rat epithelial monolayers cultured in media with PMMA extracts showed marked increases in LDH release, intracellular formaldehyde and lysosomal levels, and reductions in attached cell number and amount of E-cadherin compared with those in culture without the extracts, which were alleviated or prevented by pre-incorporation of NAC into the PMMA co-polymer. Human oral mucosal epithelial monolayer culture with PMMA extracts was higher in intracellular ROS levels and lower in total and oxidized glutathione content than the culture without the extracts. In contrast,
the culture with extracts from PMMA pre-incorporated with NAC showed a lower ROS level and a higher level of total glutathione but a similar level of oxidized glutathione compared with culture without extracts. Cells in the culture with the extracts produced higher levels of proinflammatory cytokines, such as GM-CSF, M-CSF, IL-1β, extracts p1 and MCP-3, than culture without extracts, whereas those increases were suppressed in culture with extracts from PMMA pre-incorporated with NAC. A relatively high concentration of formaldehyde was detected in the extracts, which was reduced by pre-incorporation of NAC into PMMA. The extracts from PMMA pre-incorporated with NAC exerted DPPH radial scavenging capability in contrast with the PMMA extract alone.

**Conclusion:** Pre-incorporation of NAC into self-curing PMMA co-polymer resulted in prevention of cellular damage and inflammatory response via oxidative stress in oral mucosal epithelial cells by the PMMA extracts. The mechanism was a reduction of formaldehyde elution from the PMMA co-polymer rather than a reinforcement of cellular antioxidant capability.
**Introduction**

Oral mucosa constitutes the major part of the oral cavity including gingiva, bucco and tongue. A superficial layer of oral mucosa consists of epithelial tissue that serves as a physical barrier to protect subepithelial tissue from invasion of foreign matter, such as bacteria, microparticulate and chemical substances. In addition, epithelial tissue also functions as an initiator of immunological reaction in response to invasion of antigens by production of proinflammatory cytokines to recruit and activate immune cells[1]. Oral mucosal epithelial tissue is likely to be disordered because of frequent exposure to various external stimuli.

Self-curing polymethylmethacrylate (PMMA) dental resin is widely used in prosthodontic and restorative treatments because of the advantages of versatility, user-friendliness and physical properties. However, its biological adverse effects have been underestimated. The possibility of intraoral contact mucositis in association with acrylic resin material including PMMA has been noted[2]. It has been reported that many dental staff exhibit resin-related contact dermatitis[3]. One of main components in PMMA is methylmethacrylate (MMA)[4, 5]. It is known that PMMA co-polymer elutes residual MMA over days even after polymerization. MMA causes not only direct destruction of cellular components by the reactive center, but also oxidative stress leading to apoptosis or inflammation via intracellular over-generation of reactive oxygen species (ROS)[6]. In addition, formaldehyde is also generated as by-product after hydrolysis and oxidative degradation of MMA[7], which exerts more severe cytotoxicity than that of MMA itself[7] and induces apoptotic and necrotic cell death[4, 5]. Both MMA and formaldehyde can also infiltrate into the inside of cells and disturb cellular homeostasis. In fact, it has been extensively reported that PMMA co-polymer and its extracts induce apoptosis, necrosis, inflammation and dysfunction in various types of cells, such as fibroblasts[8], dental pulp cells[9-11], osteoblasts[12] and epithelial cells[13]. Oral epithelial tissue is subject to substances eluted from PMMA co-polymer in prosthodontic and restorative treatments, in particular, such as direct repair and adjustment of denture base and provisional
restorations. Adverse effects of PMMA co-polymer on oral mucosa is of concern during prosthodontic and restorative treatment.

N-acetyl cysteine (NAC) is a thiol compound with strong antioxidant capability. NAC directly scavenges ROS with the sulhydryl base as a functional moiety[14]. NAC can also be incorporated into cells and improve intracellular redox status by functioning as a supplemental source of cellular glutathione, which serves as the principal molecule in the intracellular antioxidant system[15]. Moreover, NAC can detoxify MMA by blocking the reactive center with a sulfhydryl base[6]. It has been demonstrated that incorporation of NAC into PMMA-based resin material resulted in avoidance of intracellular ROS over-generation and prevention of apoptosis, necrosis and impairment of extracellular matrix production in dental pulp cells[10, 11], fibroblasts[8] or osteoblasts[12] on the resin substrate without deterioration of the physical properties of the PMMA co-polymer. However, the following intriguing questions remain: (1) Does incorporation of NAC into PMMA-based dental resin prevent damage and inflammatory reactions of epithelial cells induced by PMMA co-polymer? (2) How does NAC work as a detoxification agent after incorporation into the material? Clarification of these questions would pave the way for the development of biological-friendly PMMA material, leading to prevention of dental material-related health hazards in the oral cavity and an upgrade of quality of dental practice.

The purpose of this in vitro study was (1) to determine if pre-incorporation of NAC into self-curing PMMA dental resin prevents damage and inflammatory reactions of oral mucosal epithelial cells by PMMA extracts and (2) to clarify the detailed mechanism underlying detoxification of PMMA co-polymer by pre-incorporation of NAC.
Materials and Methods

Extract preparation

NAC (Sigma-Aldrich Japan, Tokyo, Japan) was prepared as a 1 mol/L stock solution by mixing a powdered form of NAC in HEPES buffer. The pH of the NAC stock solution was adjusted to 7.2. The powder and liquid components of commercial self-curing PMMA-based dental resin (Unifast III, GC, Tokyo, Japan) were mixed in a 50 ml polystyrene tube on a vibrator for 15 seconds according to the manufacturer’s instructions (powder/liquid ratio of 0.6 g/0.4 g). NAC-incorporated resin was prepared by mixing the powder and the liquid containing NAC solution. The concentration of NAC was set to 0.4% in final weight percentage of the resin co-polymer. After 5 min of polymerization, 5 ml Dulbecco’s modified Eagle’s medium: Nutrient Mixture F-12 (DMEM/F12, Life Technologies, Tokyo, Japan) or oral keratinocyte medium (CnT-24, CELLnTEC Advanced Cell Systems, Bern, Switzerland) without serum was added into the tube and incubated for 7 days at 37°C. Instead of culture basal media, deionized distilled water (ddH₂O) was used for chemical assays of the extracts (see below). After incubation, the extracts were sterilized with a 0.4-µm filter and supplemented with 10% fetal bovine serum and antibiotic-antimycotic solution for culture experiments.

Cell culture

Rat oral epithelium cells were isolated from hard palatal mucosa of 8-week-old male Sprague–Dawley rats by incubation in 0.1 units/ml collagenase/0.8 units/ml dispase solution for 60 minutes and grown in DMEM/F12 with supplements. Human normal oral epithelium cells (COSMO BIO, Tokyo, Japan) were grown in CnT-24 with growth supplements. Cells underwent two to three passages and were seeded on a 24-well polystyrene culture plate at 3.0×10⁴ cells/cm². After confirmation of 80% confluence by 7 day preculture, the monolayer culture underwent renewal of culture media with the same media or the extracts and was cultured for 24 h in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The rat cell culture protocol was approved by the Animal Research Committee of Tokyo Dental College (Protocol No. 232604).
**Evaluations of influence of resin extract on rat epithelial cellular components**

Cellular damage in rat epithelial cell culture incubated for 24 h in untreated culture media or culture media containing the extracts were evaluated by remnant cell count using a hemocytometer after detachment with 0.8 units/ml dispase and 0.25% trypsin-1 mM EDTA-4Na, light microscopic observation after methylene blue-eosin staining and quantification of lactate dehydrogenase (LDH) release and amount of E-cadherin in the culture. LDH release was measured by colorimetry of culture supernatant after the addition of LDH assay reagents (Cytotoxicity Detection Kit, Roche Diagnostics, Tokyo, Japan), which was based on the generation of dihydronicotinamide adenine dinucleotide (NADH) after dehydrogenation of lactic acid by LDH and generation of red formazan dye after reduction of tetrazolium salts by NADH. The absorbance was measured at 490nm with a microplate reader (Spectra Max M5e, Molecular Devices Japan, Tokyo, Japan). The amount of E-cadherin of attached cells was analyzed by fluorimetry of culture stained with monoclonal anti-mouse E-cadherin-phycoerythrin conjugated with fluorescein isothiocyanate (R&D Systems, Minneapolis, MN, USA). After staining, fluorescent intensity in culture was measured at excitation of 490nm and emission of 515nm with a microplate reader.

**Evaluation of influence of resin extract on intracellular environment in rat oral epithelial cells**

Invasion by resin substances into rat epithelial cells was evaluated by quantification of intracellular formaldehyde and cellular lysosomal levels and activity of cellular digestion system. Epithelial cells were lysed by freezing cyclein 300 μl of 10 mmol/l HCl solution, and the whole cell lysates were analyzed with 3 ml formaldehyde-detection reagents (Nanocolor Tube Test Formaldehyde 8, Macherey-Nagel, Düren, Germany) after mixing with 300 μl of cell lysate. The methodology was based on a purple color reaction of chromotropic acid with formaldehyde under acidic conditions. Fluorimetry of cellular lysosomal level as a measure of cellular digestion activity was performed on attached cells using fluorescence staining (LysoTracker®, Lonza Japan, Tokyo, Japan). After staining,
fluorescent intensity in culture was measured at excitation of 373nm and emission of 422nm with a microplate reader.

**Evaluation of influence of resin extract on intracellular redox status in human oral epithelial cells**

Intracellular redox status in human oral epithelial cells was evaluated by fluorimetry of intracellular ROS levels and colorimetry of cellular total and oxidized glutathione levels. Intracellular ROS on attached cells was detected with 5-(and-6)-carboxy-20,70-dichloro-fluorescein diacetate (carboxy-DCF-DA) (Life Technologies), which is absorbed intracellularly and oxidized into luminous DCF-DA by intracellular ROS. After removing supernatant and washing with D-PBS, attached cells were incubated in 1ml D-PBS containing 100μl of DCF-DA. After incubation, the culture was washed again with D-PBS and underwent the fluorescent intensity measurement at excitation of 485nm and emission of 538nm with a microplate reader. Cellular total glutathione was detected using 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) (Total Glutathione Quantification Kit, Dojindo Laboratories, Kumamoto, Japan). The amount of cellular glutathione was determined by measuring the concentration of 2-nitro-5-thiobenzoic acid resulting from a chromogenic reaction between DTNB and reduced glutathione in a glutathione recycling system. The oxidized glutathione was detected by masking of reduced glutathione with a masking reagent. The cells were lysed in 0.1M HCl solution by freezing cycle. Their intracellular concentrations were calculated based upon a parallel glutathione standard curve.

**Evaluation of influence of resin extract on inflammatory status in human oral epithelial cells**

Production of proinflammatory cytokines, such as granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), interleukin (IL)-1β and -8, monocyte chemotactic and activating factor (MCP)-1 and -3, in human oral epithelial cells in culture supernatant were quantified using the multiplex cytokine immunoassay system (Veritas, Tokyo, Japan). The supernatants were incubated with beads coated with antibodies corresponding to the
various cytokines and were developed according to the manufacturer’s instructions. Bead size and fluorescence were measured on a Luminex 200 system (Luminex Japan, Tokyo, Japan) and data were analyzed using the associated Master Plex® QT (Hitachi Solutions, Tokyo, Japan). For acquiring released concentration per cell unit of proinflammatory cytokines, the value was divided by the number of attached cells in the corresponding culture.

**Quantification of resin substances and antioxidants in the extracts**

The amounts of resin substances and antioxidants were evaluated for clarification of the mechanism underlying NAC-mediated detoxification of resin material. Formaldehyde levels in the extracts prepared with ddH2O were analyzed using the abovementioned formaldehyde detection reagents. Formaldehyde concentration was calculated according to a calibration line from 1 and 10 ppm of standard formaldehyde. Antioxidant capability in extracts was evaluated by determination of the free-radical-scavenging abilities of the extracts using 2,2-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich), a stable free radical. The purple color of DPPH ethanol solution changed to orange or yellow in proportion to the antioxidant capability of the reagent. Optical density of 1 ml DPPH solution was determined at 10 minutes after the addition of 50 μl distilled water, 2.5 mM NAC solution or the extracts from untreated or NAC-incorporated resin.

**Statistical analysis**

Values determined in the analysis were divided by cell number of the corresponding duplicate culture to calculate the individual cellular amount except for LDH and E-cadherin assays. Culture experiments were performed three times in each assay (n=3). DPPH experiment was performed three times (n=3). Quantification of formaldehyde levels in the extracts was performed once (n=1). All repeated data were analyzed with Bonferroni multiple comparisons after one-way analysis of variance to determine the differences between each group using statistical software (SPSS, standard version 16, International Business Machines Corporation, NY, USA). Statistical significance was set
Results

**Cellular damage by PMMA extract and its prevention by pre-incorporation of NAC**

Light microscopic observation revealed that the rat epithelial monolayer exhibited large voids and expansion of distances between adjacent cells after a 24-h incubation with the PMMA extracts in contrast with dense colony formation in the untreated epithelial layers. However, epithelial layers after incubation with extracts from PMMA pre-incorporated with NAC maintained cell-to-cell connection and prevented cellular detachment (Figure 1A). The number of attached cells after incubation with PMMA extracts was reduced to 30% of untreated cultures (Figure 1B). However, pre-incorporation of NAC into PMMA increased the value to 80% of that in the untreated culture. The culture after incubation with the PMMA extract showed lower cadherin levels and higher LDH release than those in the untreated culture, which was prevented by pre-incorporation of NAC into PMMA (Figure 1C and D).

**Generation of intracellular formaldehyde and lysosome by PMMA extract and its reduction by pre-incorporation of NAC**

Intracellular formaldehyde levels in rat epithelial monolayer culture increased by 3.5-fold after a 24-h incubation with the PMMA extracts compared with those in the untreated cultures (Figure 1E), whereas there was no significant difference between the untreated culture and culture with incubation with extracts from PMMA pre-incorporated with NAC. Cellular lysosomal levels were 3-fold greater in the culture after incubation with the extracts than that in the untreated culture (Figure 1F). However, pre-incorporation of NAC into PMMA markedly reduced the cellular lysosomal elevation by the extracts.

**Oxidative stress by PMMA extracts and intracellular antioxidant reinforcement by NAC**
Both total glutathione and oxidized glutathione levels in human oral epithelial monolayer culture were reduced after a 24-h incubation with the PMMA extracts to almost half of those in the untreated culture (Figure 2A and B). In contrast, incubation with extracts from PMMA pre-incorporated with NAC markedly increased intracellular total glutathione levels compared with those in the untreated culture (Figure 2A). There were no significant differences in oxidized glutathione levels between the untreated culture and culture with incubation with extracts from PMMA pre-incorporated with NAC (Figure 2B). Intracellular ROS levels were markedly increased in the cells after a 24-h incubation with the PMMA extracts, which was reduced by pre-incorporation of NAC into PMMA to a lower value than that in the untreated culture (Fig. 2C).

**Epithelial inflammatory response to PMMA extracts and its prevention by pre-incorporation of NAC**

Human epithelial cell monolayer culture incubated with PMMA extracts for 24 h increased released concentration per cell unit of proinflammatory cytokine, such as GM-CSF, M-CSF, IL-1β, IL-8, MCP-1 and MCP-3 (Figure 3). Pre-incorporation of NAC into PMMA reduced all of released concentration per cell unit of proinflammatory cytokines tested as compared to those in the culture after incubation in the PMMA extracts. Except for GM-CSF, the levels after exposure to the extracts from PMMA pre-incorporated with NAC were equivalent to those in the untreated culture.

**Formaldehyde concentration and free radical scavenging capability in extracts**

The purple color of the solution was obviously denser after dropping of PMMA extracts than after dropping of extracts from PMMA pre-incorporated with NAC (Figure 4A, image). The purple density of the solution added with extracts from PMMA pre-incorporated with or without NAC was between 10 ppm and 1 ppm formaldehyde or 1 ppm formaldehyde and distilled water, respectively. Colorimetric determination revealed that the concentration of formaldehyde was 1.0 or 4.0 ppm in the extracts from PMMA pre-incorporated with or without NAC, respectively (Figure 4A, histogram).
The purple DPPH solution turned yellow after the addition of NAC solution (Fig. 4B, image), whereas the color of the solution remained unchanged by the addition of distilled water or PMMA extract. However, addition of extracts from PMMA pre-incorporated with NAC led to discernible discoloration of the DPPH solution. Optical density of DPPH solution was lower with the addition of extracts from PMMA pre-incorporated with NAC or 2.5mM NAC solution than the solution containing distilled water or the PMMA extract (Figure 4B). There was no significant difference in the value between NAC pre-incorporated PMMA extract and NAC solution (Figure 4, histogram).

**Discussion**

In this study, the extracts from polymerized PMMA-based dental resin reduced the attached cell number and amount of E-cadherin and increased LDH release in oral epithelial monolayer culture. Light microscopic observation revealed that the PMMA extracts made large voids and expansion of distances between adjacent cells in epithelial monolayer culture. All of these results indicated that the extracts caused a breakdown of cellular components resulting in both cellular detachment and loss of cell-to-cell connection. In addition, relatively high concentrations of formaldehyde were detected in the extracts, which was one of representative decomposition products of MMA through hydrolysis or oxidative degradation. Previous investigation exhibited the amounts of formaldehyde and MMA release in the extracts from an autopolymerized PMMA dental resin were approximately 2 ppm and 137 ppm for 4 days, respectively[7]. Over 80% or 50% of gastrointestinal L cells was dead after exposure to 1.5 or 500 ppm of formaldehyde or MMA, respectively[7]. Release of these chemicals from co-polymer gets a peak at first day with a gradual reduction with immersion days, but continues for 30 days after polymerization[16]. In this study, 4.0 ppm of formaldehyde was detected in the PMMA extracts which was made by 7 days incubation in distilled water. Therefore, the PMMA extracts used in this study might contain more amounts of MMA, as well as
formaldehyde, than the previous investigation. Moreover, the PMMA extracts prepared according to the protocol in the previous experiment [10], was much severe condition for culture experiment.

It is well known that MMA caused direct destruction of cellular components [17]. The beta carbon of the double bond in the molecular structure of MMA shows electrophilic reactivity and reacts to nucleophilic centers of proteins and phospholipids via Michael addition [17]. In addition, formaldehyde enables to cause cellular damage by degenerative change of protein structure[18]. Therefore, formaldehyde and MMA in the extracts might directly destroy cellular components by reacting with proteins and phospholipids.

Glutathione maintains the intracellular redox system by repeating the oxidation-reduction cycle in cytoplasm. Reduced glutathione is transformed into the oxidized form after eliminating physiologic ROS generated by the process of cellular metabolism. Oxidized glutathione is transformed into the reduced form by glutathione reductase[19]. This cycle maintains intracellular redox balance. Hyperproduction of ROS causes collapse of redox balance so-called “oxidative stress” by glutathione depletion and apoptosis induction[17, 19] leading to destruction of cellular components. Reduction of total and oxidized glutathione levels and an increase of intracellular ROS levels in human oral mucosal epithelial monolayer culture were observed after exposure to the extracts; this suggested that the PMMA extracts might induce apoptosis via oxidative stress, as well as direct cellular destruction, on oral mucosal epithelial cells.

The processes of generation of cellular ROS by the PMMA extracts are wide-ranging and remain to be fully elucidated. In this study, exposure to the extracts resulted in detection of formaldehyde even in the inside of cells and elevation of cellular lysosome levels in oral mucosal epithelial cells. Both formaldehyde and MMA are lipophilic and theoretically can gain entry into the inside of cells[20] where many types of degradative enzymes are abundant and the cellular digestion system is activated
by foreign matter[21]. It is well known that excessive stress to mitochondria and endoplasmic reticulum (ER) by various external stimuli, such as ultraviolet radiation and chemical substances, result in the leakage of ROS from these cellular metabolic organelles[22, 23]. In this study, formaldehyde and MMA in the extracts entered into epithelial cells as foreign matter and might cause mitochondrial damage and ER stress, leading to excessive generation of intracellular ROS. Clarification of the influence of MMA on cellular organelles is of great interest for future research.

Under this severe condition, pre-incorporation of NAC into PMMA co-polymer alleviated the destruction of the oral mucosal epithelial culture, cellular redox imbalance and production of proinflammatory cytokines after exposure to the extracts. There are several candidate pathways for detoxification of PMMA material by NAC incorporation. Not only reduction of cellular ROS levels, but also marked increase of total glutathione levels were observed in human oral mucosal epithelial cells after exposure to the extracts from PMMA pre-incorporated with NAC. In addition, the extracts from NAC pre-incorporated resin scavenged DPPH radicals. NAC undergoes rapid cellular uptake and is intracellularly transformed into reduced glutathione[15]. These facts suggest that NAC molecules eluted from PMMA co-polymer pre-incorporated with NAC and reinforced the antioxidant capability of epithelial cells.

However, the levels of oxidized glutathione in the culture after exposure to extracts from PMMA pre-incorporated with NAC were not different from the levels in the culture without extracts. In addition, both the intracellular formaldehyde and lysosomal levels were much lower after exposure to extracts from PMMA pre-incorporated with NAC than from the untreated material. These indicated that cells exposed to extracts from PMMA pre-incorporated with NAC might not suffer from oxidative stress. Furthermore, formaldehyde levels in the extracts from PMMA pre-incorporated with NAC were obviously low. The sulfhydryl group in NAC molecule plays a main role in the antioxidant capability of NAC and functions as a direct ROS scavenger and a
nucleophilic center reacting with beta carbon of the double bond of MMA[17]. In fact, previous reports have demonstrated that the mixture of MMA monomer and NAC lost both cytotoxicity and antioxidant capability[6]. Pre-incorporation of NAC might prevent direct cellular destruction by the extract, possibly by binding of the sulfhydryl group in NAC to the reactive center of residual MMA monomer in PMMA material. These were estimated that pre-incorporation of NAC reduced the elution of MMA from PMMA themselves. Therefore, neutralization of the residual MMA rather than reinforcement in resistance against oxidative stress may have contributed to the detoxification of PMMA co-polymer by NAC in this study.

Contact of epithelial tissue with exogenous irritant substances and antigens threatens to evoke irritant contact dermatitis (ICD) or mucositis[24]. The pathogenesis of dermatitis has been fully investigated and is most likely similar to that of mucositis. Keratinocytes, the majority of epithelial tissue, are activated by irritant substances and subsequently produce proinflammatory cytokines, such as IL-1, -8 and GM-CSF[24, 25]. These cytokines are associated with onset and development of ICD by induction of inflammatory cell infiltration, vascular hyperpermeability and excessive ROS generation[21, 26]. In addition to these cytokines, MCP-1, -3 and M-CSF activate antigen presenting cells in epithelial and connective tissue, such as Langerhans cells or macrophages, and lead to further development of ICD or, possibly, allergic contact dermatitis (or mucositis)[1, 27]. In this study, human oral mucosal epithelial cells after exposure to the PMMA extracts increased production of proinflammatory cytokines, such as IL-1β, IL-8, MCP-1, -3, M-CSF and GM-CSF. Levels of released cytokine concentration per cell unit was equivalent or more to those observed in articles dealing with human peripheral blood mononucleated cells[28], monocytes[29] or RAW264.7 macrophages[30] activated with hemagglutinin or lipopolisaccaride. The amounts of these cytokine productions from epithelial cells were reduced by pre-incorporation of NAC into PMMA. These observations estimated that application of the antioxidant NAC eliminates proinflammatory cytokine production potentially relevant to onset and development of irritant or allergic contact mucositis
from oral epithelial tissue. Further investigation concerning the pathogenesis of epithelial inflammation by PMMA co-polymer and its prevention by application of antioxidants will be of great interest for future research.

Conclusion

The extracts from self-curing PMMA co-polymer exerted cellular damage, such as cellular injury and induction of proinflammatory cytokine production, via oxidative stress on oral mucosal epithelial cells. Pre-incorporation of NAC into PMMA co-polymer eliminated the cellular damage. The mechanism was a reduction of formaldehyde elution rather than reinforcement of cellular antioxidant capability along with NAC elution from the co-polymer.

Acknowledgments

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Figure 1.
Figure 2.

A

Total glutathione level (µM)

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Oxidized glutathione level (µM)

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C

Intracellular ROS level (fluorescence intensity)

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Figure 4.
Legends

**Figure 1.** Light microscopic images (A) and results of cell number (B), amount of E-cadherin (C), LDH release (D), intracellular formaldehyde level (E) and lysosomal level (F) of rat oral epithelial cultures after 24-h incubation with a culture media with or without (w/o) the extracts from PMMA with (w/) or without (w/o) pre-incorporation of NAC. Data are shown as the mean ± SD (n = 3). *p < 0.05 (Bonferroni multiple comparison)

**Figure 2.** Results of amount of total glutathione (A) and oxidized glutathione (B), and intracellular ROS level (C) of human oral epithelial cultures after 24-h incubation with a culture media with or without (w/o) the extracts from PMMA with (w/) or without (w/o) pre-incorporation of NAC. Data are shown as the mean ± SD (n = 3). *p < 0.05 (Bonferroni multiple comparison)

**Figure 3.** Results of concentration per cell unit of proinflammatory cytokine released from human oral epithelial cultures after 24-h incubation with a culture media with or without (w/o) the extracts from PMMA with (w/) or without (w/o) pre-incorporation of NAC, quantified by multiplex cytokine immune assay for GM-CSF (A), M-CSF (B), IL-1β (C), IL-8 (D), MCP-1 (E) and MCP-3 (F). Data are shown as the mean ± SD (n = 3). *p < 0.05 (Bonferroni multiple comparison)

**Figure 4.** (A) Upper images showing purple color intensity of formaldehyde-detection reagents after a droplet of distilled water (DW), 1 and 10 ppm formaldehyde solutions and extracts from PMMA with (w/) or without (w/o) NAC pre-incorporation. Bottom histogram shows the concentration of formaldehyde in extracts from PMMA with (w/) or without (w/o) pre-incorporation of NAC (n = 1). (B) Upper images and bottom histogram showing disappearance of purple color and optical density of 1 ml DPPH solution after a 50 μl droplet of distilled water (DW), 1 M NAC solution and extracts from PMMA with (w/) or without (w/o) pre-incorporation of NAC, respectively. Data are shown as the mean ± SD (n = 3). *p < 0.05 (Bonferroni multiple comparison)