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Alleviation of Commercial Collagen Sponge- and Membrane-Induced Apoptosis and Dysfunction in Cultured Osteoblasts by an Amino Acid Derivative

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Purpose: The objectives of this *in vitro* study were to determine whether the commercial collagen material used in bone augmentation procedures induces oxidative stress-mediated adverse effects on the viability and function of osteoblasts and to determine whether N-acetyl cysteine (NAC), an antioxidant amino acid derivative, can alleviate these effects. **Materials and Methods:** Commercial collagen sponge (Collaplug) and membrane (BioGide) were treated with NAC. Rat calvaria-derived osteoblasts were directly seeded on these materials with or without NAC pretreatment. Cytotoxic evaluation was performed by flowcytometric cell viability assay, confocal laser microscopic analysis of attached cell morphology and reactive oxygen species (ROS) localization, and alkaline phosphatase staining. **Results:** Cell viability was less than 40% on both collagen sponge and membrane 24 hours after seeding and increased to 50% with NAC pretreatment. Cell death was characterized by apoptosis. Colonization of attached cells was sparse on the untreated sponge and membrane on day 1, and the cells were round, small, and filled with intense and closely packed intracellular ROS. In contrast, NAC-pretreated material had dense cell colonies consisting of well-spread osteoblasts and fully developing cytoskeleton and cellular processes with little ROS generation. On day 7 of culture, NAC-pretreated collagen sponge and membrane yielded an expanded alkaline phosphatase-positive area occupying 60% and 80% of the surface area, respectively, whereas the untreated collagen materials had limited alkaline phosphatase activity (7% or less). **Conclusions:** Commercial collagen sponge and membrane induced considerable cell death, impaired initial function, and generated extraordinary intracellular ROS in attached osteoblasts, whereas NAC pretreatment substantially ameliorated these effects. The potential benefits of NAC's detoxifying capacity on bone regeneration using collagen matrix materials in an animal model should be confirmed with further study. INT J ORAL MAXILLOFAC IMPLANTS 2010;25:939-946

Key words: antioxidant, biocompatibility, guided bone regeneration, oxidative stress, socket preservation

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Collagenous matrix products such as collagen sponge or membrane, which are generally derived from allogeneic or xenogeneic sources, are frequently used in socket preservation^{1,2} or guided bone regeneration procedures^{3,4} for dental implant site development. Such collagen matrix devices are usually placed at the superficial region of the bone-grafted site to avoid spread of subjacent graft materials^{1,5,6} and provide a barrier against soft tissue invasion into the bone-forming region.^{7,8} Moreover, they must function as a bioabsorbable scaffold to allow ingrowth⁷ and replacement by newly formed tissue,^{1,5} especially bone tissue.⁹ Therefore, it is crucial for the collagen matrix materials to be biocompatible with bone tissue/cells for a bone augmentation procedure to produce successful outcomes. However,

detailed information about the osteocompatibility of commercially available collagen matrix materials has not been fully provided,⁹ in contrast with their well-proven compatibility with mucosal tissue.⁷

Osteoblast viability, attachment, adhesion, and proliferation, along with extracellular matrix production and matrix mineralization capability on a scaffold, are essential for osteogenesis on any biomaterial.¹⁰ Adverse responses of osteoblasts to biomaterials lead to delayed bone formation or impaired bone volume/quality. Therefore, osteocompatibility is the biologic property of not inhibiting viability and function of osteoblasts,¹¹ which can be strictly investigated by *in vitro* testing.¹²

Recent culture studies reported that currently available biomaterials may have biologically adverse effects on various types of cells. Neutrophils died after coming into contact with expanded polytetrafluoroethylene (e-PTFE),¹³ and mitochondrial activity decreased in periodontal ligament fibroblasts and in an osteosarcoma cell line after touching e-PTFE membrane.¹⁴ Titanium alloy activated monocytes and macrophages, leading to damage of osteoblasts,¹⁵ whereas hydroxyapatite particles increased inflammatory cytokine production from osteoblasts by contact stimuli.¹⁶ Polymethyl methacrylate resin, which is categorized as a biotolerant material, nearly completely suppressed cellular viability, extracellular matrix production, and related gene expression of fibroblasts and dental pulp cells.^{17,18} Although the mechanism is still unknown, this material-induced cytotoxicity is thought to be associated with oxidative stress on cells resulting from an excessive generation of intracellular reactive oxygen species (ROS).¹⁹

N-acetyl cysteine (NAC) is an antioxidant amino acid derivative. It can be incorporated into a cell and deacetylated into L-cysteine, which is a glutathione precursor.²⁰ Glutathione plays a central role in the intracellular ROS elimination system.²⁰ NAC can rescue cells from oxidative stress by preventing or recovering the depleted intracellular glutathione level.²¹ Therefore, NAC may detoxify collagen matrix materials if they cause oxidative stress-related cytotoxicity in osteoblasts. In fact, NAC prevents suppression of cell viability and function in fibroblasts and dental pulp cells cultured on polymethyl methacrylate-based dental resin,^{18,22,23} which is recognized to cause oxidative stress on cells.^{22,23} The objectives of the present *in vitro* study were: (1) to determine whether commercially available collagen sponge or membrane negatively impacts the viability and function of cultured osteoblasts in conjunction with oxidative stress and, if so, then (2) to determine whether NAC detoxifies the collagenous matrix materials and prevents cell dysfunction and death.

MATERIALS AND METHODS

Collagen Specimens and NAC Preparation

Collagen sponge (Collaplug, Zimmer Dental) and membrane (BioGide, Osteohealth) were purchased and trimmed into small pieces that were uniform in superficial area (44.2 mm²) and thickness (0.3 mm). Each specimen was placed on a culture-grade polystyrene 48-well plate. An NAC stock solution was prepared by dissolving NAC powder (Sigma-Aldrich) in HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer (1 mol/L stock, pH 7.2). The NAC treatment solution was prepared by mixing the NAC stock solution and alpha-modified Eagle's medium (α -MEM) (Gibco/Invitrogen) in a volume ratio of 1 to 9. Immediately before cell seeding, 50 μ L of the NAC treatment solution or α -MEM alone was permeated into the collagen material substrate. It was preliminarily confirmed that both types of collagen specimen maintained enough structural integrity to undergo analyses, even after immersion in a culture medium for 7 days.

Osteoblastic Cell Culture

Osteoblasts were derived from parietal and frontal bones of 8-week-old male Sprague-Dawley rats. Calvaria were stripped of sutures, periosteum, and dura mater. After washing with 1% phosphate-buffered solution (PBS) (MP Biomedicals), the parietal and frontal bones were dissected into small pieces (< 1 mm²) and digested with 0.25% collagenase (Sigma) for 12 hours. The liberated cells were collected and plated on 100-mm plastic tissue culture dishes with α -MEM supplemented with 15% fetal bovine serum, 50 μ g/mL ascorbic acid, 10⁻⁸ mol/L dexamethasone, 10 mmol/L Na- β -glycerophosphate, and an antibiotic-antimycotic solution containing 10,000 units/mL penicillin G sodium, 10,000 mg/mL streptomycin sulfate, and 25 mg/mL amphotericin B. The cells were incubated in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. At 80% confluence, the cells were detached using 0.25% trypsin-1 mmol/L ethylenediaminetetraacetic acid (EDTA)-4Na and seeded onto polystyrene substrates with or without 50 μ L of the NAC solution supplied in a culture media. The collagen specimens pretreated with α -MEM or NAC were seeded in the 48-well culture plate at a density of 4×10^4 cells/cm² in 0.25 mL of the osteoblastic media without ascorbic acid. 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.

Detection of Cell Viability and Apoptosis

Cell viability and apoptosis in the culture were evaluated 24 hours after seeding by flow cytometry using

an apoptosis detection kit (Annexin V-FITC Kit, BD Bioscience). All of the floating cells and attached cells in culture were collected into a tube. After a supernatant of culture was collected, cultured collagen specimens were transferred to a new culture plate. Subsequently, the collagen substrates were gently rinsed twice with Dulbecco PBS (D-PBS) and the attached cells were detached using 0.25% trypsin–1 mmol/L EDTA–4Na. After centrifugation, the cells were stained with annexin V and propidium iodide (PI). This method is based on the binding properties of annexin V to phosphatidylserine and on the DNA-intercalating capability of PI. The intensity of PI staining (y-axis) was plotted against fluorescein isothiocyanate intensity (x-axis). The percentages of viable cells (quadrant 3: annexin V [–]/PI [–] cells), apoptotic cells (quadrant 2: annexin V [+]/PI [+] cells; quadrant 4: annexin V [+]/PI [–] cells), and necrotic cells (quadrant 1: annexin V [–]/PI [+] cells) were determined from the plotting pattern of the cell population. The selected collagen specimens were fixed with 2.0% glutaraldehyde and dried for scanning electron micrographic (SEM) observation to confirm the absence of cell remnants on the substrates.

Quantification of Attached Cell Numbers

The number of attached cells in cultures was evaluated using calcein acetoxymethyl ester (calcein AM) (Cell Counting Kit-F, Dojindo Molecular Technologies), which is hydrolyzed to calcein by intracellular esterase. On day 1, cultured collagen specimens were transferred to a new culture plate. The attached cells were gently rinsed twice with D-PBS and detached using 0.25% trypsin–1 mmol/L EDTA–4Na. After centrifugation, the cells were incubated in calcein AM solution in D-PBS for 30 minutes at 37°C. After the calcein AM solution was removed, the cells were gently rinsed with Dulbecco PBS again. Calcein fluorescence intensity was measured using a fluorescence plate reader at 535 nm (excitation at 485 nm) against D-PBS as a blank. SEMs of the selected cultures were obtained to confirm the absence of cell remnants on the substrates.

Measurement of Intracellular ROS Level

The amount of intracellular ROS production in attached cells was quantified by fluorimetry with 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCF-DA) (Invitrogen), which is absorbed intracellularly and oxidized into luminous DCF-DA by intracellular ROS. On day 1, cultured collagen specimens were transferred to a new culture plate. The cells were gently rinsed twice with D-PBS and subsequently trypsinized. After centrifugation, the cells were incubated in carboxy-DCF-DA solution in D-PBS

for 15 minutes at 37°C. After the carboxy-DCF-DA solution was removed, the cells were gently rinsed with D-PBS again. DCF-DA fluorescence intensity was measured using a fluorescence plate reader at 520 nm (excitation at 488 nm) against D-PBS as a blank. The selected cultures were subjected to SEM to confirm the absence of cell remnants on the substrates.

Cell Morphology, Morphometry, and Intracellular ROS Visualization

After 24 hours of culture, the osteoblasts were fixed in 10% formalin and stained with fluorescent dyes, rhodamine phalloidin (actin filament red color, Molecular Probes) for cytoskeleton staining, and carboxy-DCF-DA for intracellular ROS detection. Confocal laser scanning microscopy was used to examine cell morphology and cytoskeletal arrangement and to visualize ROS production in the cells. A quantitative assessment of cell morphometry (cell area, perimeter, and Feret's diameter) was performed using an image analyzer (Image J, National Institutes of Health).

Alkaline Phosphatase Staining

On day 7, the cultures were washed twice with Hank's solution and incubated with 120 mmol/L Tris buffer (pH 8.4) containing 0.9 mmol/L naphthol AS-MX phosphate and 1.8 mmol/L fast red TR for 30 min at 37°C. The stained images were analyzed for alkaline phosphatase (ALP) –positive area, defined as: $[\text{stained area}/\text{total substrate area}] \times 100$ (%) using Image J.

Statistical Analysis

Three independent cultures were evaluated in each group, except for the cell morphometry experiment in which six samples were evaluated, and there were at least three replicates in each experiment using different cell batches. All repeated measurements were expressed as means \pm standard deviations, and the existence of significant differences ($P < .05$) among the experimental groups was determined by one-way analysis of variance. When needed, the Student-Neuman-Keuls multiple-comparisons test was used post hoc.

RESULTS

NAC Rescues Osteoblasts from Collagen Matrix Material-Induced Apoptosis

Compared to the cell viability on polystyrene (90%), the untreated collagen sponge and membrane showed less cell viability ($< 40\%$; $P < .01$, Student-Neuman-Keuls test) (Fig 1) at 24 hours after seeding; however, NAC pretreatment increased cell viability on the collagen

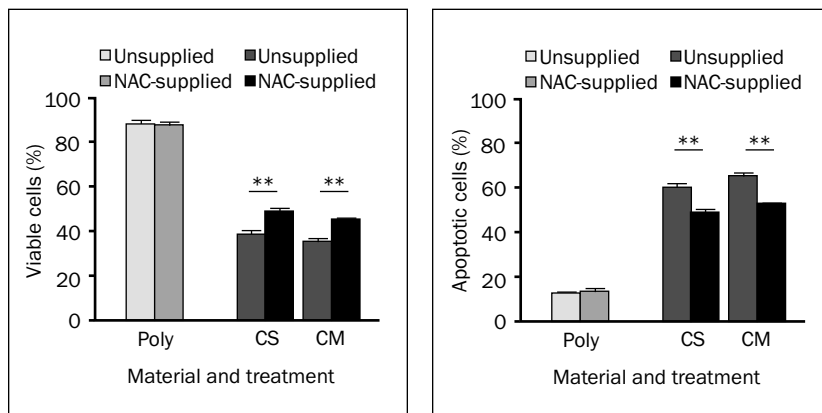


Fig 1a Results of cell viability and apoptosis analysis using flow cytometry with dual staining for annexin V and PI in osteoblastic cultures grown on collagen matrix materials with or without NAC pretreatment or on polystyrene with or without NAC supplementation in a culture media 24 hours after seeding. Poly = polystyrene; CS = collagen sponge; CM = collagen membrane.

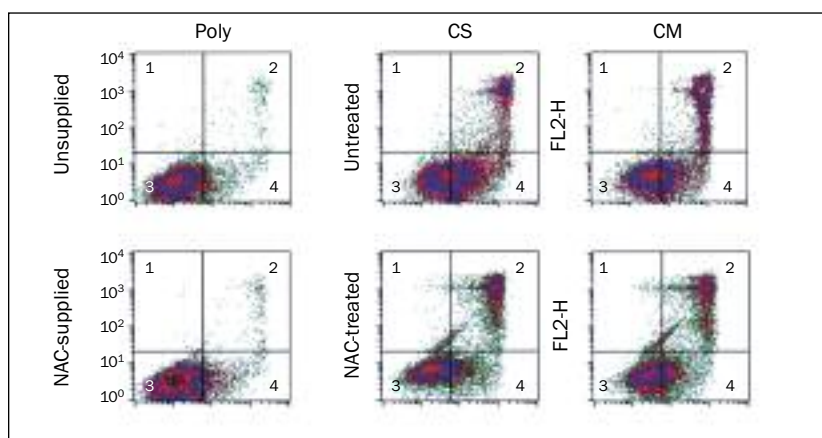


Fig 1b Flow cytometric images are shown on the top half of each box, and the percentages of viable cells (quadrant 3) and apoptotic cells (quadrants 2 and 4) are shown on the right. Data shown in histograms at left are means ± SDs (n = 3). ***P* < .01: significant difference between untreated and NAC-treated for the same type of collagen material (Student-Neuman-Keuls test). Poly = polystyrene; CS = collagen sponge; CM = collagen membrane.

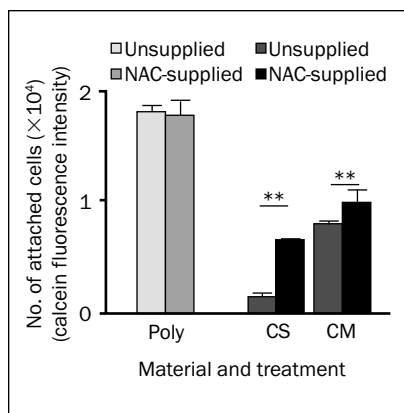


Fig 2 Results of attached cell number quantified by calcein fluorophotometry in osteoblastic cultures on collagen matrix materials with or without NAC pretreatment or on polystyrene with or without NAC supplementation in a culture media at day 1. Data are means ± SDs (n = 3). ***P* < .01, significant difference between untreated and NAC-treated on the same type of collagen material (Student-Neuman-Keuls test). Poly = polystyrene; CS = collagen sponge; CM = collagen membrane.

materials by 1.3 times (*P* < .01). More than 60% of osteoblasts were apoptotic on the untreated collagen materials, in contrast with 12% on polystyrene (*P* < .01). There was 50% or less apoptosis on both types of collagen materials that had been pretreated with NAC (*P* < .01). NAC supplementation to polystyrene culture did not result in any significant changes in the percentage of viable and apoptotic cells.

NAC Increases the Number of Attached Cells on Collagen Matrix Materials

On day 1 of culture, compared to polystyrene, 10% and 40% fewer osteoblasts attached to the collagen sponge and membrane, respectively (Fig 2) (*P* < .01). However, NAC-pretreated collagen sponge and membrane had 3.5 times and 1.2 times more attached cells, respectively, than the untreated materials (*P* < .01). There was no significant difference in the number of attached cells between polystyrene cultures with or without NAC supplementation.

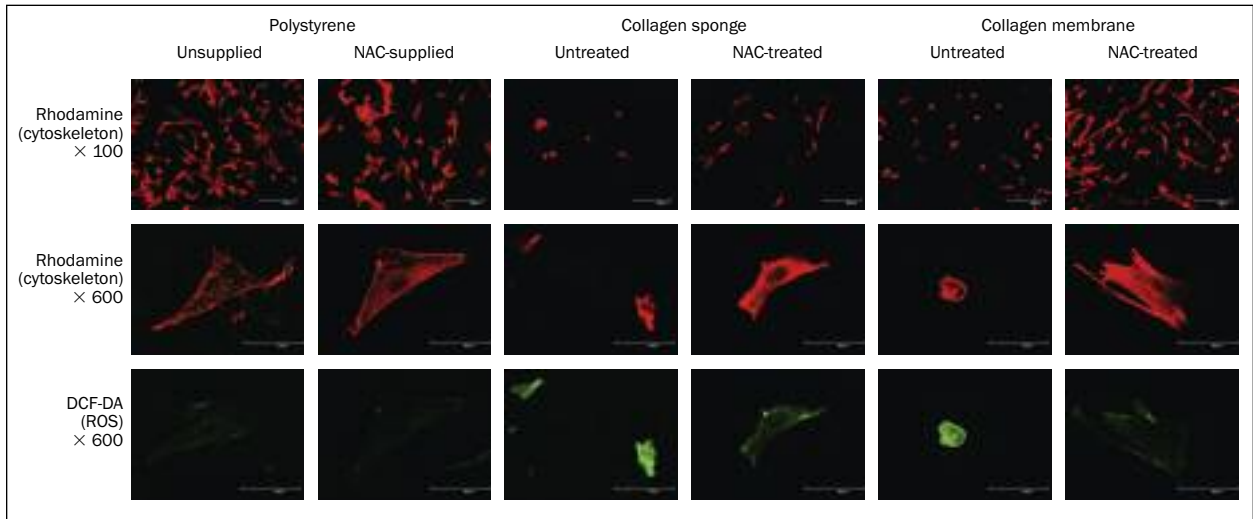
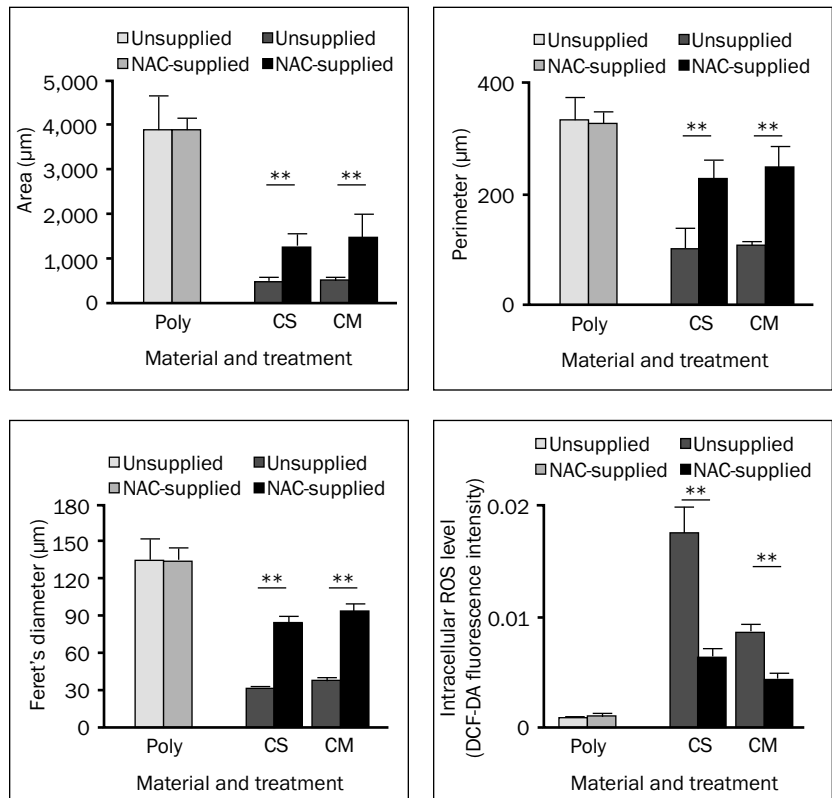


Fig 3a Representative dual-stained confocal laser microscopic images using rhodamine for actin filaments (red) and DCF-DA for intracellular ROS (green) (top panels) of cultures on collagen matrix materials with or without NAC pretreatment or on polystyrene with or without NAC supplementation in a culture media 24 hours after seeding. Colonial appearance of osteoblastic culture (top panels, magnification $\times 100$), cell morphology, and intracellular ROS distribution in the same cells (top panels, magnification \times) are shown.

Fig 3b Cell morphometric evaluations were performed by image analysis. Data are means \pm SDs ($n = 6$). Also shown are the results of intracellular ROS level evaluated by DCF-DA fluorophotometry on day 1 of osteoblastic cultures on collagen matrix materials with or without NAC pretreatment or on polystyrene with or without NAC supplementation in a culture media. Data are means \pm SDs ($n = 3$). $**P < .01$: significant difference between untreated and NAC-treated for the same type of collagen material (Student-Neuman-Keuls test). Poly = polystyrene; CS = collagen sponge; CM = collagen membrane.



NAC Relieves Impaired Osteoblastic Cell Adhesion and Marked Intracellular ROS Production on Collagen Matrix Materials

There were fewer attached osteoblasts on the untreated collagen matrix materials than on polystyrene 24 hours after seeding (Fig 3a). The osteoblasts cultured on polystyrene with and without

NAC supplementation were fully expanded, developed cytoskeletal filaments and cellular processes, and they exhibited little intracellular ROS, whereas the cells cultured on the untreated collagen matrix materials were round, small, and filled with dense ROS (Fig 3a). NAC pretreatment visibly increased osteoblastic adhesion on the collagen materials (Fig 3a). Relatively wide,

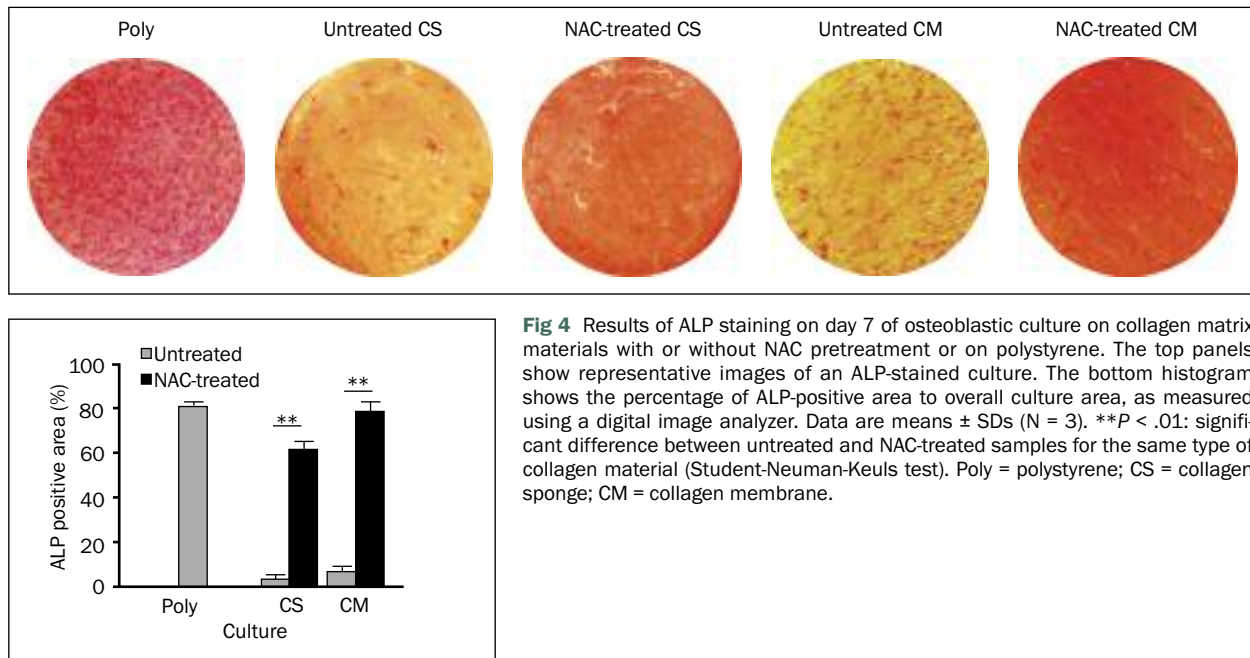


Fig 4 Results of ALP staining on day 7 of osteoblastic culture on collagen matrix materials with or without NAC pretreatment or on polystyrene. The top panels show representative images of an ALP-stained culture. The bottom histogram shows the percentage of ALP-positive area to overall culture area, as measured using a digital image analyzer. Data are means \pm SDs (N = 3). ** $P < .01$: significant difference between untreated and NAC-treated samples for the same type of collagen material (Student-Neuman-Keuls test). Poly = polystyrene; CS = collagen sponge; CM = collagen membrane.

large, and stretched cells with weak and sparse ROS were found on the NAC-treated collagen materials (Fig 3a). The area, perimeter, and Feret's diameter of the cells of the untreated collagen matrix materials was less than one third of that measured on polystyrene cultured with and without NAC (Fig 3b). NAC-pretreated collagen materials exhibited values that were two to three times greater for these parameters than the untreated materials.

Carboxy-DCF-DA fluorescence quantification revealed that intracellular ROS production in the day 1 cultures was 18 and nine times greater on cells attached to the untreated collagen sponge or membrane, respectively, than on polystyrene (Fig 3b) ($P < .01$). NAC reduced the collagen material-elevated ROS production down to less than half of that on the untreated collagen materials ($P < .001$). There was no significant difference in intracellular ROS levels between polystyrene cultures with or without NAC supplementation.

NAC Prevents Suppressed ALP Activity of Osteoblasts on Collagen Matrix Materials

Low ALP activity was detected on the collagen sponge and membrane in day 7 cultures (Fig 4, top panels), whereas an extensive ALP-positive area was observed on polystyrene. The percentage of ALP-positive area was only 4% and 7% on the collagen sponge and membrane, respectively, whereas it was over 80% on polystyrene (Fig 4, histogram) ($P < .01$). In contrast, NAC-pretreated collagen matrix products had intensive and wide-ranging ALP-positive areas (Fig 4, top panels). More than 60% of the collagen

sponge area became ALP positive following NAC pretreatment (Fig 4, histogram); this was 16 times greater than that on the untreated collagen sponge ($P < .01$). Moreover, NAC-pretreated collagen membrane manifested 80% of ALP-positive area, which was 11 times greater than that on the original collagen membrane ($P < .01$) and was level equivalent to that measured on polystyrene ($P > .05$).

DISCUSSION

The present study demonstrated that commercially available collagen sponge and membrane hindered the viability, attachment, and adhesion of osteoblasts as well as the expression of the osteoblastic phenotype. Fewer than 40% of the osteoblasts survived and more than 60% of the cells went into apoptosis on untreated collagen matrix materials. There were few attached osteoblasts on the untreated collagen material, and they were impaired in cell stretch, formation of cellular processes, and cytoskeletal development, which is essential cellular behavior not only for establishing cell adhesion, but also for proliferation, extracellular matrix production, and mineralization.²⁴ In fact, ALP activity, an early marker of osteoblastic differentiation, was inhibited on the untreated collagen materials. This suppression of initial osteoblastic responses on commercial collagenous products suggests that currently used collagen materials must be improved to increase osteocompatibility.

The adverse biologic effects of the collagen materials on osteoblasts were accompanied by an extraordi-

nary generation of intracellular ROS. Although the underlying mechanisms are still unknown, several studies have reported that currently available biomaterials can evoke negative cellular responses in association with oxidative stress.^{15,19} Oxidative stress results from the distortion of the cellular redox balance caused by excess intracellular ROS production.²⁰ Excessive generation of intracellular ROS is triggered by external stimuli such as ultraviolet light, ionizing radiation, chemical compounds, and extracellular ROS.²⁰ Consequently, the cells undergo oxidizing attacks through lipid peroxidation, DNA strand breaks, and a dysregulation of the cellular signaling pathways, which induces apoptosis and cellular dysfunction.²⁰

Collagen matrix products are biodegradable and water soluble, and some physicochemical phenomena of degrading collagen-based material are suspected to be a cause of oxidative stress. One is a release of chemical compounds from the collagen material. Collagen matrix products are generally extracted from allogeneic or xenogeneic organs such as the Achilles tendon, dermis, and dura mater. Following antigen treatment to remove telopeptides from the original collagen fibers, the collagen is cross-linked to enhance structural stability of the collagen polymer and to control its biodegradability.²⁵ Some studies have shown that the biocompatibility of collagen-based material is influenced by the cross-linking method.²⁵ Glutaraldehyde is the most widely used chemical cross-linking agent because of its well-proven efficacy for collagen stabilization.²⁵ For instance, the collagen sponge used in the present study (Collaplug) was cross-linked with glutaraldehyde. However, glutaraldehyde forms a large-molecular-weight polymer once it contacts water, and the polymers are retained within the collagen structure; this results in continual release of glutaraldehyde during biodegradation,²⁶ which contributes to prolonged toxic effects following tissue implantation.²⁵ In fact, glutaraldehyde cross-linked collagen material is poorly compatible with endothelial cells, fibroblasts, osteoblasts, and other types of cells.²⁷ In the present study, glutaraldehyde may have eluted from the collagen sponge (Collaplug) and become involved in the toxic effects on osteoblasts through oxidative stress, which might contribute to a greater reduction in attached cell numbers and ALP activity on the glutaraldehyde cross-linked collagen sponge than that on the noncross-linked collagen product (BioGide).²⁸

In addition, contact interaction between the cell and degrading collagen substrate may be suspected as one mechanism underlying collagen material-induced oxidative stress. A previous organ culture study indicated that the surface collagen bands of an enzymatically fragmented collagen tissue

scaffold developed low-density reduced tensibility of attached fibroblasts, as compared to intact collagen matrix with a compacted structure, resulting in increased intracellular ROS production and negative impacts on fibroblastic protein synthesis.²⁹ The extracellular matrix influences cellular behavior through multiple mechanisms, including integrin-mediated cellular contacts that control cell shape and intracellular mechanical forces and provide cellular structural integrity and physical support to tissues.²⁹ In the present study, noncross-linked collagenous membrane (BioGide)¹ caused oxidative stress in osteoblasts with oxidative stress as a result of unlinking of the material's surface structure. Mechanisms underlying collagen material-induced oxidative stress and cytotoxicity should be clarified in future research.

The cytotoxic effects of collagen materials on osteoblasts were alleviated by NAC treatment before seeding. There were significantly more viable and attached cells on the NAC-treated material than on the untreated material. Osteoblastic cell adhesion was firmly established and subsequent ALP expression was markedly activated on NAC-treated collagen materials. In addition, extraordinary ROS elevation in osteoblasts was prevented by NAC. These results demonstrated that NAC detoxified the collagen matrix materials and encouraged osteoblasts to accomplish the initial stage of osteogenesis on collagen material, which implies a potential for enhanced or accelerated bone formation on materials treated with NAC. It has been well proven that NAC can detoxify acrylic resin materials and prevent suppressed viability and function.^{7,17,22,30}

NAC is an antioxidant amino acid derivative and acts as a cellular glutathione supplement. Therefore, NAC functions not only as a direct ROS scavenger but also as a cytoprotective agent to compensate for intracellular redox imbalance.^{20,21,30} Moreover, NAC may act directly on cellular molecules. It is hypothesized that NAC affects intracellular redox-sensitive transcriptional factors^{20,21} that play a key role in the cellular signaling pathways mediating cellular survival and differentiation.²⁰ Some studies indicate that NAC protects various types of cells from toxic reagent-induced cell death or loss of function by modulating transcriptional factors.³¹ It will be of great interest to explore in detail how NAC prevents the adverse biologic effects of collagenous matrix material on osteoblasts.

In bone-regenerating therapy, it is necessary for collagenous matrix products to function not only as a scaffold to support bone formation but also to act as a barrier against mucosal tissue invasion into the grafted region. However, collagenous matrix materials continue to require improvement in their capacity

to shield areas from soft tissue.⁷ A previous culture study demonstrated that NAC can restrict overgrowth of mucosal cells on collagen sponge and membrane, indicating successful control of fibrogenic proliferation in addition to its ameliorating effect on oxidative stress.³⁰ Moreover, this implies NAC's multifunctional potential, namely, its selective affinity to osteoblasts rather than fibroblasts in favor of bone augmentation procedures using collagen matrix materials. Although further *in vivo* confirmation is needed, the present study warranted biologic amelioration of collagenous matrix products by NAC and provided important information for developing a next-generation collagen material for bone-regenerating therapy.

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