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Improvement in osteoblastic cellular response to commercial collagen membrane and demineralized freeze-dried bone by amino acid derivative: an *in vitro* study

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Short title: Ameliorated osteocompatibility in collagenous materials by NAC

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PURPOSE: The objectives of this *in vitro* study were 1) to determine whether commercially available collagen membrane (CM) or human demineralized freeze-dried bone (DFDB) particles adversely affected viability or function in cultured osteoblasts through oxidative stress; and, if so, 2) to determine whether N-acetyl cysteine (NAC) successfully prevented loss of viability and dysfunction in osteoblasts.

MATERIALS AND METHODS: Rat calvaria-derived osteoblasts were seeded onto polystyrene and commercially available CM (Cytoplast®) or DFDB (DynaGraft™) with or without pretreatment with NAC solution. Osteoblastic response was evaluated by flow cytometric cell viability assay, measurement of attached viable cell number, quantification of reactive oxygen species (ROS) and alkaline phosphatase (ALP) staining.

RESULTS: The percentage of viable cells on CM was less than 50% at 24 hr after seeding. However, this increased to 70% by pretreatment with NAC. Numbers of attached osteoblasts on DFDB remained at 60% the level of that on polystyrene at 24 hr after seeding, but increased to up to 90% the level of that on polystyrene with NAC pretreatment. Although collagen materials increased intracellular ROS generation by 1.5-5.0 times that with polystyrene, this was significantly reduced by NAC pretreatment. The percentage of ALP positive area was consistently 7 % or less on CM and DFDB at days 7 and 14, which was restored by NAC pretreatment up to 60 % or more.

CONCLUSIONS: Commercially available CM and DFDB impaired osteoblastic viability and function and markedly increased intracellular ROS, indicating an oxidative stress-mediated negative impact on osteoblasts. Pretreatment with NAC substantially alleviated these cytotoxic effects.

Keywords: biomaterials; bone substitute; guided tissue regeneration; material sciences; wound healing

Introduction

Collagen matrix originating from allogeneic or xenogeneic tissue is widely applied as bone substitute and barrier membrane. Representative of such collagenous biomaterials are human demineralized freeze-dried bone (DFDB) and bovine or porcine connective tissue-derived collagen membrane, which are frequently used in alveolar bone augmentation/regeneration procedures, including sinus floor elevation (Handschel, et al. 2009, Hassani, et al. 2009, Tawil & Mawla 2001) and guided bone regeneration (GBR) (Avera, et al. 1997, Zitzmann, et al. 1997, Zubillaga, et al. 2003). These materials maintain the space needed for bone regeneration to take place, preventing invasion by soft tissue and 3-dimensional structural collapse (Schroeder & Brown 1999, Strietzel, et al. 2006). Moreover, they act as a scaffold to support functional expression of osteogenic cells, thus enabling formation of new bone (Herold, et al. 2002, Hile, et al. 2005, Xiao, et al. 2003, Zhao, et al. 2009). Therefore, the osteocompatibility of these materials will affect the quantity, quality and speed of bone formation, all of which are linked to clinical outcome.

Osteogenesis on a biomaterial results from a sequential process of osteoblastic cellular activity, which includes osteoblast survival, attachment, adhesion, proliferation, extracellular matrix production and matrix mineralization (Cooper, et al. 1998). Such cellular activity can be accurately assessed by a culture method (Coelho, et al. 2000). The current definition of biocompatibility emphasizes the biological properties of a material in determining cellular response and subsequent tissue formation (Williams 2003). Essential to the osteocompatibility of a material is its non-interference with osteoblastic viability or function. However, previous reports suggested that osteoblastic cellular response to some types of commercially available collagen matrix materials has a room to be improved and remains to be fully clarified (Becker, et al. 1994, Becker, et al. 1995, Bilir, et al. 2007, Carinci, et al. 2007, Piattelli, et al. 1996, Takata, et al. 2001, Wang, et al. 2002).

Recent culture studies have demonstrated that currently available biomaterials exert a biologically

adverse effect on various types of cell. Coming into contact with expanded polytetrafluoroethylene (e-PTFE) membrane resulted in neutrophil cell death (Nadzam, et al. 2000) and decreased mitochondrial activity in periodontal ligament fibroblasts and osteosarcoma cell line (Alpar, et al. 2000). Titanium alloy activated monocytes and macrophages, leading to damage of osteoblasts (Kalbacova, et al. 2007). Contact with hydroxylapatite particles increased inflammatory cytokine production in osteoblasts (Lenz, et al. 2009). Polymethyl methacrylate (PMMA) resin, a biotolerant material, induced massive cell death and almost complete suppression of cellular function in fibroblasts, dental pulp cells and osteoblasts (Kojima, et al. 2008, Tsukimura, et al. 2009). Such adverse cellular reaction to biomaterials may be associated with excessive generation of intracellular reactive oxygen species (ROS), resulting in oxidative stress, although this remains to be confirmed (Tsaryk, et al. 2007, Tsukimura, Yamada, Aita, Hori, Yoshino, Chang-Il Lee, Kimoto, Jewett & Ogawa 2009).

N-acetyl cysteine (NAC) is a membrane-permeable anti-oxidant amino acid derivative that is metabolized into L-cysteine, a precursor of glutathione, which is an essential molecule in the intracellular ROS elimination system (Schweikl, et al. 2006). The functional moiety of NAC, its sulfhydryl group, can directly scavenge free radicals and toxic compounds (Novitskiy, et al. 2006, Thibodeau, et al. 2001, Yamada & Ogawa 2009). The antioxidative function of NAC protects cells from oxidative stress by preventing disruption of intracellular redox balance (Zafarullah, et al. 2003). This suggests that if collagenous bone graft materials negatively influence osteoblasts via oxidative stress, then NAC might improve their osteocompatibility. In earlier studies, we demonstrated that NAC restored suppressed cellular viability and differentiation in fibroblasts, dental pulp cells and osteoblasts on PMMA-based dental resin (Kojima, Yamada, Paranjpe, Tsukimura, Kubo, Jewett & Ogawa 2008, Sato, et al. 2009, Tsukimura, Yamada, Aita, Hori, Yoshino, Chang-Il Lee, Kimoto, Jewett & Ogawa 2009), a representative oxidative-inducing biomaterial, in culture and animal models (Kojima, Yamada, Paranjpe, Tsukimura, Kubo, Jewett & Ogawa 2008, Sato, Ueno, Kubo,

Suzuki, Tsukimura, Att, Yamada, Hori, Maeda & Ogawa 2009). The objectives of this *in vitro* study were 1) to determine whether commercially available collagen membrane (CM) or DFDB particles adversely affected viability or function in cultured osteoblasts through oxidative stress; and, if so, 2) to determine whether NAC successfully prevented loss of viability and dysfunction in osteoblasts.

Materials and methods

Collagen membrane, collagenous bone substitute and NAC preparation

Type I bovine CM (Cytoplast® RTM, Sybron Implant Solutions, Orange, CA, USA) was cut into small pieces, uniform in shape and with a surface area of 44.2 mm^2 each. Human DFDB particles (DynaGraft-D^{TM} putty, Keystone Dental, Burlington, MA, USA) were combined to form particle clusters of 0.01 g in weight. Each sample was placed in a culture-grade polystyrene 48-well plate. An NAC stock solution was prepared by dissolving NAC powder (Sigma-Aldrich, St. Louis, MO, USA) in HEPES buffer (1 mol/L stock, pH 7.2). The NAC-treatment solution was prepared by mixing the NAC stock solution with alpha-modified Eagle's medium (α-MEM, Gibco BRL Div. of Invitrogen, Gaithersburg, MD, USA) in a volume ratio of 1 to 9. Immediately before cell seeding, the collagen material on the culture plate was soaked in 50 μl NAC-treatment solution or α-MEM alone. It was confirmed that the collagen membrane retained sufficient integrity after immersion in culture medium for 7 days to allow subjection to analysis.

Rat osteoblastic culture

Co-culture model with physical contact between cells and material

Calvarial osteoblasts were derived from the parietal or frontal bones of 8-week-old male Sprague-Dawley rats. Calvariae were stripped of sutures, periosteum and dura mater. After washing with 1% phosphate buffered solution (PBS; MP Biomedicals, Solon, OH, USA), the parietal and frontal bones were dissected into small pieces (<1 mm^2) and digested with 0.25% collagenase (Sigma) for 12 hr. Liberated cells were collected and plated onto 100-mm plastic tissue culture dishes

containing α-MEM supplemented with 15% fetal bovine serum, 50 μg/mL ascorbic acid, 10^{-8} M dexamethasone, 10 mM Na-ß-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. Cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. At 80% confluence, the cells were detached using 0.25% trypsin-1 mM EDTA-4Na and seeded onto polystyrene, untreated collagen material, or NAC-treated material (Fig. 1A) at a density of 3×10^4 cells/cm^2 in 1.0 mL osteoblastic media without ascorbic acid.

Co-culture model without physical contact between cells and material

To evaluate the biological effect of DFDB on osteoblasts under non-physical contact conditions, a non-contact co-incubation model using a culture insert chamber with a submicron porous bottom (0.04-μm pore filter) (Millicell, Millipore, Bedford, MA, USA) was used. The chamber, containing 0.04 g DFDB and 250 μl α-MEM or NAC-treatment solution, was suspended above a 12-well polystyrene plate on which rat osteoblasts were seeded (Fig. 1B) to a density of 3×10^4 cells/cm^2 in 1.0 mL osteoblastic media without ascorbic acid. The bottom of the chamber was submerged at a distance of 3.0 mm from the bottom of the culture plate. This allowed the osteoblasts to be cocultured with the DFDB on the polystyrene but without physical contact. Polystyrene cultures under the chamber containing none or 20 μL/mL of methyl methacrylate (MMA) (Endurance MV, DePuy Orthopaedics, Warsaw, IN) were made as positive or negative control culture for this culture model, respectively.

The cells were cultured for up to 14 days and 70 μl of fresh medium was added for 3 days. The study protocols were approved by the University of California Los Angeles Chancellor's Animal Research Committee.

Detection of osteoblastic viability and death

Cell viability and apoptosis in the culture on polystyrene and collagen membrane were evaluated at 24 hr after seeding by flow cytometry using an apoptosis detection kit (Annexin V-FITC Kit, BD Bioscience, San Jose, CA, USA). Floating and attached cells in the culture were collected into a tube. After collecting culture supernatant, cultured collagen specimens were transferred to a new culture plate. Subsequently, the collagen substrates were gently rinsed twice with D-PBS and attached cells detached using 0.25% trypsin-1 mM 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 the FITC intensity (x-axis). The percentages of viable cells (quadrant 3: annexin V [−]/PI [−] cells), apoptotic cells (quadrant 2: annexin V [+]/PI [+] cells and 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. Both apoptotic and necrotic cells were regarded simply as “dead cells” for convenience in this study. The selected collagen specimens were fixed with 2.0% glutaraldehyde and dried for scanning electron microscopy (SEM) to confirm the absence of cell remnants on the substrates.

Quantification of viable cell number

To evaluate the influence of DFDB and collagen membrane on osteoblastic viability and attachment, Calcein-AM fluorescent staining (Cell Counting Kit-F, Dojindo Molecular Technologies, Rockville, MD, USA) was employed. Calcein-AM is intracellularly incorporated into viable cells and hydrolyzed to calcein by intracellular esterase. After discarding the supernatant from osteoblastic cultures at 24 hr after seeding on untreated polystyrene, collagen membrane, DFDB and polystyrene under the culture insert containing none or DFDB were gently rinsed twice with D-PBS and subjected to trypsinization with 0.25% trypsin-1 mM EDTA-4Na. After centrifugation, the cells were incubated in Calcein-AM solution in D-PBS for 30 min at 37°C. After removing the Calcein-AM solution, the cells were gently rinsed with D-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. SEM of the selected cultures was carried out to confirm the absence of cell remnants on the substrates.

Measurement of intracellular ROS level

The amount of intracellular ROS production in attached cells at 24 hr after seeding on untreated polystyrene, collagen membrane or polystyrene under the culture insert containing none or DFDB was quantified by fluorometry with 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCF-DA) (Invitrogen, Gaithersburg, MD, USA), which is absorbed intracellularly and oxidized into luminous DCF-DA by intracellular ROS. Immediately before fluorescent staining, cultured collagen specimens were transferred to a new culture plate. After discarding the supernatant, osteoblastic cultures were gently rinsed twice with D-PBS and subjected to trypsinization with 0.25% trypsin-1 mM EDTA-4Na. After centrifugation, the cells were incubated in carboxy-DCF-DA solution in D-PBS for 15 min at 37°C. After removing the carboxy-DCF-DA solution, 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.

Alkaline phosphatase staining

On days 7 and 14, osteoblastic cultures on polystyrene, collagen membrane or DFDB were washed twice with Hank's solution and incubated with 120 mM Tris buffer (pH 8.4) containing 0.9 mM naphthol AS-MX phosphate and 1.8 mM fast red TR for 30 min at 37°C. The images were analyzed to determine area of positive staining for alkaline phosphatase (ALP), defined as: [(stained area/total substrate area) × 100] (%) using ImageJ.

Statistical analysis

For all experiments, three independent cultures were evaluated in each group (n = 3) using different batch of the cells and there were at least three replicates in each experiment. All repeated measurements were expressed as means ±SD and significant differences ($p < 0.05$) among the experimental groups were evaluated by one-way or two-way ANOVA with a post hoc Bonferroni comparison.

Results

NAC protects osteoblasts from inhibitions of cell viability and attachment induced by collagen membrane

Flow cytometric analysis revealed that the mean percentage of viable cells on CM without NAC pretreatment at 24 hr after seeding was 47% in contrast with 93% on polystyrene ($p < 0.01$, Bonferroni) (Fig 2A). However, NAC pretreatment increased the percentage of viable cells on CM to 70%. Likewise, the percentage of dead cells showed a marked increase on CM as compared to that on polystyrene, and this was significantly reduced by pretreating the material with NAC ($p < 0.01$, Bonferroni).

The number of attached viable osteoblasts on CM at 24 hr after seeding was 42% less than that on polystyrene (Fig 2B) ($p < 0.01$, Bonferroni), which increased to 75% of that on polystyrene by pretreatment with NAC ($p < 0.01$, Bonferroni).

NAC prevents influence of DFDB on osteoblastic viability and attachment

The number of attached viable osteoblasts at 24 hr after seeding was 35% less on DFDB than on polystyrene (Fig 3) ($p < 0.01$, Bonferroni). Pretreatment with NAC, however, increased this percentage by up to 85% of that on polystyrene. A 20% reduction in number of attached viable cells was evoked on polystyrene under a culture insert containing DFDB as compared to that on polystyrene under a culture insert containing none ($p < 0.01$, Bonferroni). However, this reduction in

osteoblastic viability and attachment on polystyrene under DFDB was prevented by NAC pretreatment, as shown by a Calcein intensity level equivalent to that on untreated polystyrene. Polystyrene culture under a culture insert containing MMA showed a 87% reduction in number of attached viable cells as compared to that on polystyrene under a culture insert with no reagent.

NAC alleviates collagen material-induced marked increase in intracellular ROS level

DCDF-DA fluorescence quantification revealed that intracellular ROS production in viable osteoblasts at 24 hr after seeding was 6 times on untreated CM than on polystyrene (Fig. 4A). The value in osteoblasts cultured on polystyrene under a porous bottomed-culture insert containing DFDB was 2.5 times greater than that on polystyrene under a culture insert with no reagent, which was a level equivalent to that on polystyrene under a culture insert with MMA (Fig. 4B). However, this elevation in intracellular ROS induced by collagen matrix material was significantly reduced by pretreatment with NAC ($p < 0.05$) (Fig. 4A and B).

NAC restores suppressed ALP activity in osteoblasts on collagen matrix materials

In contrast to an extensive ALP-stained culture on polystyrene, very little ALP activity was detected on CM or DFDB on day 7. Pretreatment with NAC, however, resulted in a marked restoration of ALP activity (Fig. 5, top panel). The percentage of the ALP-positive area was greater than 90% on polystyrene, but less than 2% on CM or DFDB (Fig. 5, histogram) ($p < 0.01$). However, both CM and DFDB yielded an up to 60% ALP-positive area with NAC pretreatment. The percentage of ALP positive area on CM or DFDB at day 14 was higher than that at day 7, but the value still remained 7% or less. In contrast, collagen materials pretreated with NAC showed over 30 % in the percentage of ALP positive area even at day 14. Especially, the percentage of ALP positive area on DEDB pretreated with NAC increased from day 7 to day 14. On day 14, polystyrene culture showed a reduction in the percentage of ALP positive area as compared to that at day 7.

Discussion

The results of the present study revealed that viability and function were suppressed in osteoblasts cultured on commercially available CM or DFDB, with less than 50% of osteoblasts surviving on the former. Numbers of viable attached osteoblasts were markedly reduced on DFDB. Cell survival and attachment are essential as the initial steps in proliferation, extracellular matrix production and matrix mineralization (Garcia & Reyes 2005). Furthermore, ALP activity, essential in the initial stage of mineralization of the bone matrix, was suppressed both on CM and DFDB, as shown by only 2% at day 7 and 7% or less even at day 14 in the percentage of ALP positive area. This indicates the need to inhibit the induction of such adverse effects on osteoblastic function by collagenous bone graft materials if the osteocompatibility of such materials is to be improved.

Moreover, DFDB exerted a biologically negative impact, even under conditions where cells could only have been exposed to substances of < 0.4 μm in size such as MMA used in this study and where there was no direct physical contact between cells and material. For example, a 20% reduction in number of attached viable cells was evoked on polystyrene under DFDB as compared to that on polystyrene alone. This indicates that DFDB induces osteoblastic cell death not only by contact stimuli, but also by release of substances capable of exerting a cytotoxic effect.

The adverse biological effects exerted on osteoblasts by the collagen materials used in this experiment were accompanied by a marked elevation in intracellular ROS. Oxidative stress has been shown to disturb the cellular signaling pathways involved in cell viability, proliferation and differentiation (Chiarugi, et al. 2003, Griendling & FitzGerald 2003, Schweikl, Spagnuolo & Schmalz 2006). Oxidative stress leading to cell death and dysfunction can be evoked by external physic-chemical stimuli such as ultraviolet light, ionizing radiation, extracellular ROS, chemical compounds (Schweikl, Spagnuolo & Schmalz 2006, Tsukimura, Yamada, Aita, Hori, Yoshino,

Chang-Il Lee, Kimoto, Jewett & Ogawa 2009) and certain biomaterials (Kalbacova, Roessler, Hempel, Tsaryk, Peters, Scharnweber, Kirkpatrick & Dieter 2007, Tsaryk, Kalbacova, Hempel, Scharnweber, Unger, Dieter, Kirkpatrick & Peters 2007).

Without exception, commercially available collagen materials are derived from human or animal organ tissue, which means that they must undergo some physicochemical or enzymatic treatment during their manufacturing process. Current procedures for DFDB involve the use of ethanol to remove lipids and hydrochloric acid to remove the mineral components of bone. Generally, commercial collagen membrane originates from allogeneic or xenogeneic tissues such as the Achilles tendon, dermis or dura mater. The telopeptides, which function as major antigenic sites, and intermolecularly cross-linked sites in the original collagen filament are removed during its production, with subsequent cross-linking of fibers to enhance the structural stability of the collagen polymer and control its biodegradability using a physical, chemical or enzymatic method (Arem 1985). Some studies indicated that this process influenced the cytocompatibility of the collagenous material (Arem 1985). For example, collagenous material cross-linked with glutaraldehyde showed a continual release of glutaraldehyde with biodegradation (Jorge-Herrero, et al. 1999), which contributes to prolonged toxic effects following tissue implantation on endothelial cells, fibroblasts, osteoblasts and other types of cell (Arem 1985, Gough, et al. 2002). The released agents from DFDB, that caused the cellular irritation, should be identified in future research.

In addition, structural collapse of the collagenous matrix may result in oxidative stress on attached cells. Culture of fibroblasts on an enzymatically fragmented collagen tissue scaffold with low-density surface collagen bands resulted in an increase in intracellular ROS production as compared to with culture of cells on intact collagen matrix with a compact structure (Fisher, et al. 2009). Collagen matrix allows cell attachment by direct bonding through cell adhesion membrane molecules, integrins (Jokinen, et al. 2004, White, et al. 2004), and by indirect connection via

noncollagenous extracellular matrix such as fibronectin, which has several adhesion function domains to cells and matrix (Sekiguchi & Hakomori 1980). Binding of integrin adhesion receptors to extracellular matrix components activates the signaling pathways which direct osteoblast survival, proliferation and differentiation (Garcia & Reyes 2005, Gronthos, et al. 2001, Jikko, et al. 1999, Mizuno & Kuboki 2001). Generally, commercially available CM is biodegradable and water-soluble. Therefore, the inhibition of osteoblastic cell attachment and elevation of intracellular ROS contributing to cell death and loss of ALP activity on the CM in this experiment may have resulted from degradation of the membrane's surface structure. Identification of which agents were responsible for the cytotoxic effects observed here would be of great interest in future study as this would help clarify the mechanism underlying the negative response of osteoblasts to currently available collagenous graft materials.

NAC alleviated the cytotoxic effects of CM and DFDB on osteoblasts in the present study. The percentage of viable cells on CM increased from 47% to 70% with NAC pretreatment. Inhibition of cellular viability and attachment by DFDB was restored by NAC to levels comparable with those in untreated polystyrene culture. Moreover, NAC prevented a suppression of ALP activity on the materials. For instance, the percentage of ALP positive area on NAC-treated DFDB was over 45% in contrast with under 2% on original DFDB, which increased up to 65% at day 14. ALP activity on NAC-treated CM was intensive and extensive at day 7 (60% in the percentage of ALP positive area) and subsequently waned at day 14 as well as polystyrene culture, which suggested that differentiation status of cultured osteoblasts proceeded to a next stage after getting over with an initial phenotype expression (Cooper, Masuda, Yliheikkila & Felton 1998). These results indicate that NAC improves the osteoblastic cytocompatibility of collagenous bone graft materials, enhancing the osteoblastic viability and initial function required to accomplish osteogenesis on and around collagen materials, which would improve bone regeneration mediated by NAC-pretreated materials *in vivo*. Indeed, application of NAC detoxified polymethyl methacrylate-based bone cement and

markedly enhanced the contact area and biomechanical interfacial strength between material and new bone tissue in a rat femur bone marrow space model (Tsukimura, Yamada, Aita, Hori, Yoshino, Chang-Il Lee, Kimoto, Jewett & Ogawa 2009).

NAC is an antioxidant amino acid derivative and membrane-permeable compound. Its chemical and biochemical properties allow it to function not only as a direct ROS scavenger, but also as a glutathione supplement to compensate for intracellular redox imbalance (Sato, Ueno, Kubo, Suzuki, Tsukimura, Att, Yamada, Hori, Maeda & Ogawa 2009, Schweikl, Spagnuolo & Schmalz 2006, Tsukimura, Yamada, Aita, Hori, Yoshino, Chang-Il Lee, Kimoto, Jewett & Ogawa 2009, Zafarullah, Li, Sylvester & Ahmad 2003). NAC prevented marked elevation of intracellular ROS in osteoblasts by exposure to collagen materials. Relief of oxidative stress on osteoblasts by NAC may explain why NAC yielded an improvement in the osteocompatibility of the collagen materials used in the present study. Moreover, it is hypothesized that NAC can directly modulate intracellular signaling pathways by affecting the redox-sensitive transcriptional factors (Schweikl, Spagnuolo & Schmalz 2006, Zafarullah, Li, Sylvester & Ahmad 2003) that are involved in the regulation of cellular pro-inflammatory reaction, survival, proliferation and differentiation (Katoh, et al. 2005, Marshall, et al. 2000, Sarkar & Fisher 2006, Schweikl, Spagnuolo & Schmalz 2006). Some culture studies showed that NAC protected dental pulp cells from toxic reagent-induced cell death or loss of function by affecting such transcriptional factors (Paranjpe, et al. 2007). It will be of great interest in future study to explore the extracellular and intracellular mechanisms underlying the enhancement of the osteocompatibility of collagen materials by NAC.

Multiple interactions among various types of cells such as osteoblast, periosteal cell, endothelial cell, fibroblast and immunocyte are involved in bone formation on/around biomaterial in local tissue. The present study showed only negative responses of osteoblasts to collagenous materials and its alleviation by application of anti-oxidant amino acid derivative, NAC. As well as the effects of NAC

in animal model, the involvement of collagen materials and NAC in cellular interaction networks will be an intriguing target for future study. The results of the present study demonstrated that NAC improved the osteocompatibility of CM and DFDB. These findings would provide a new perspective on and important information for the development of innovative collagenous bone graft materials.

Conclusion

Commercially available collagen matrix-based bone graft material tested, a collagen membrane and demineralized freeze-dried bone particles, induced a loss of viability and a suppression of ALP activity on cultured rat calvaria-derived osteoblasts in this study. These negative cellular responses to the materials may be associated with oxidative stress. Under the limitation of this culture experimental condition, pretreatment of these materials with anti-oxidant amino acid derivative, NAC, resulted in an improvement of the biological adverse effects of these materials on osteoblasts. These results provided the further interest for exploring the effects of NAC in animal model.

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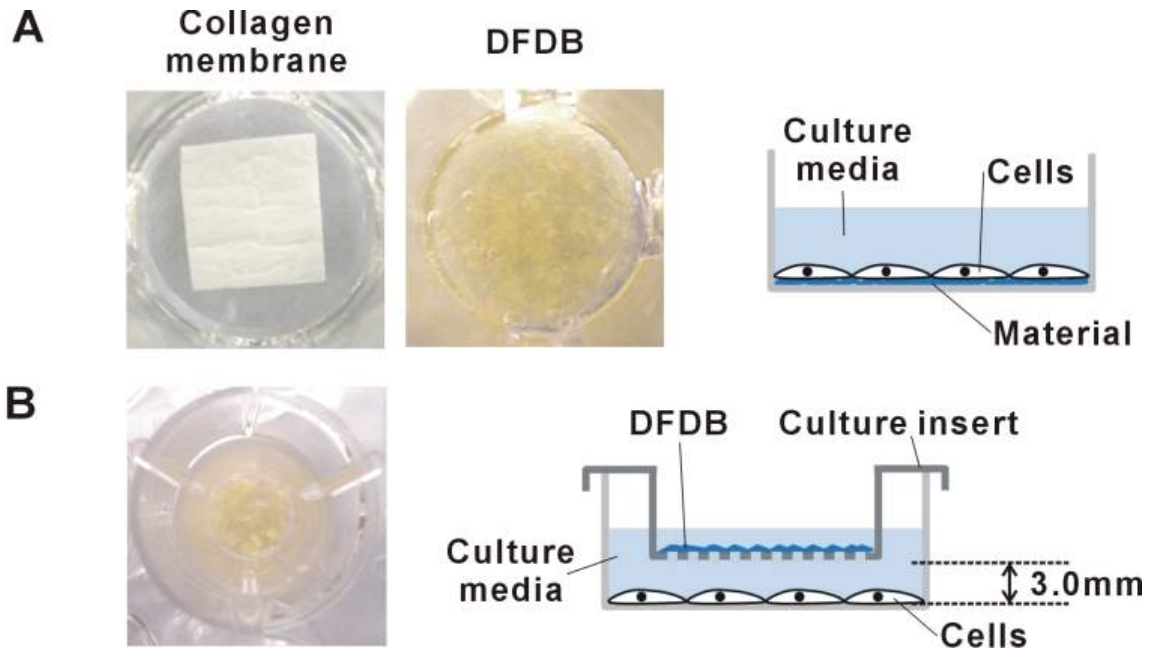
A
Collagen membrane
DFDB
Culture media
Cells
Material
B
DFDB
Culture insert
Culture media
3.0mm
Cells

Figure. 1

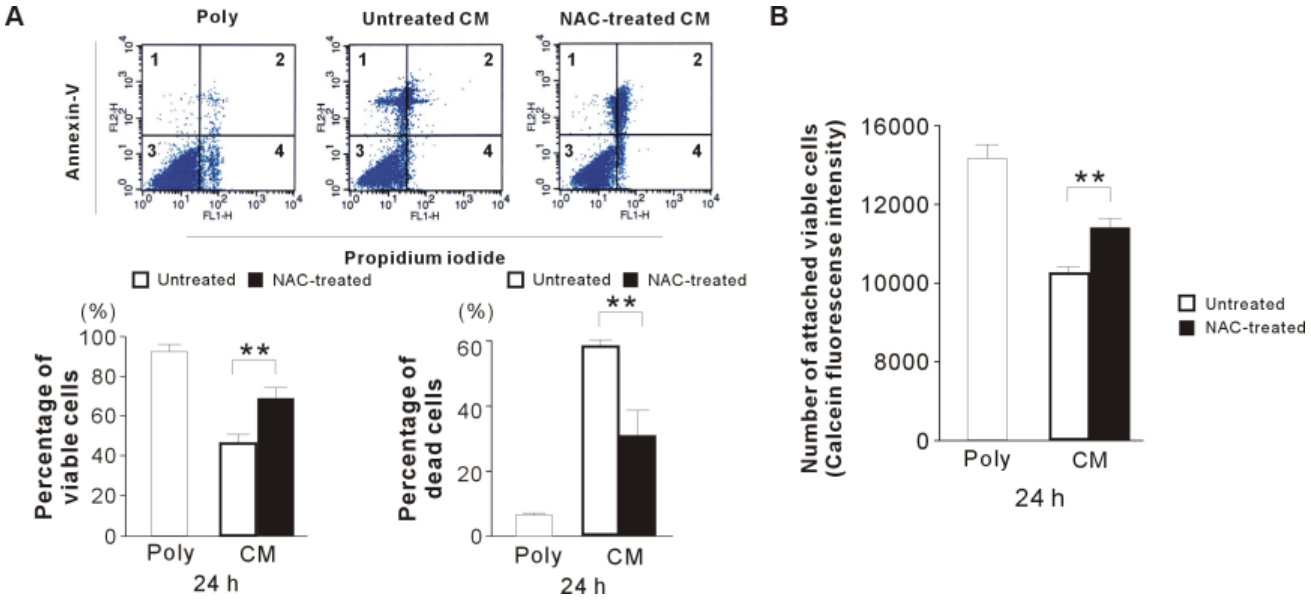
A
Poly
Untreated CM
NAC-treated CM
Annexin-V
FL2-H
FL1-H
1
2
3
4
Propidium iodide
Untreated
NAC-treated
(%)
Percentage of viable cells
Percentage of dead cells
**
Poly
CM
24 h
B
Number of attached viable cells (Calcein fluorescense intensity)
16000
12000
10000
8000
0

Figure. 2

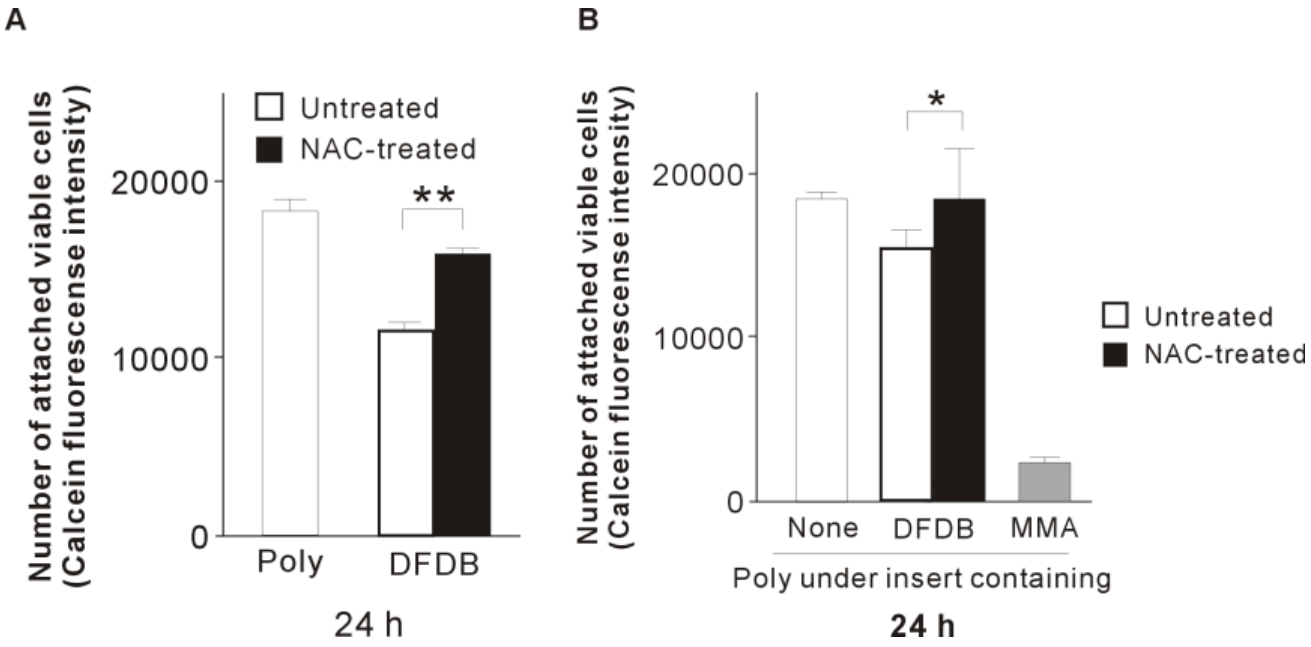
A
Number of attached viable cells (Calcein fluorescense intensity)
20000
10000
0
Untreated
NAC-treated
**
Poly
DFDB
24 h
B
Number of attached viable cells (Calcein fluorescense intensity)
20000
10000
0
*
Untreated
NAC-treated
None
DFDB
MMA
Poly under insert containing
24 h

Figure. 3

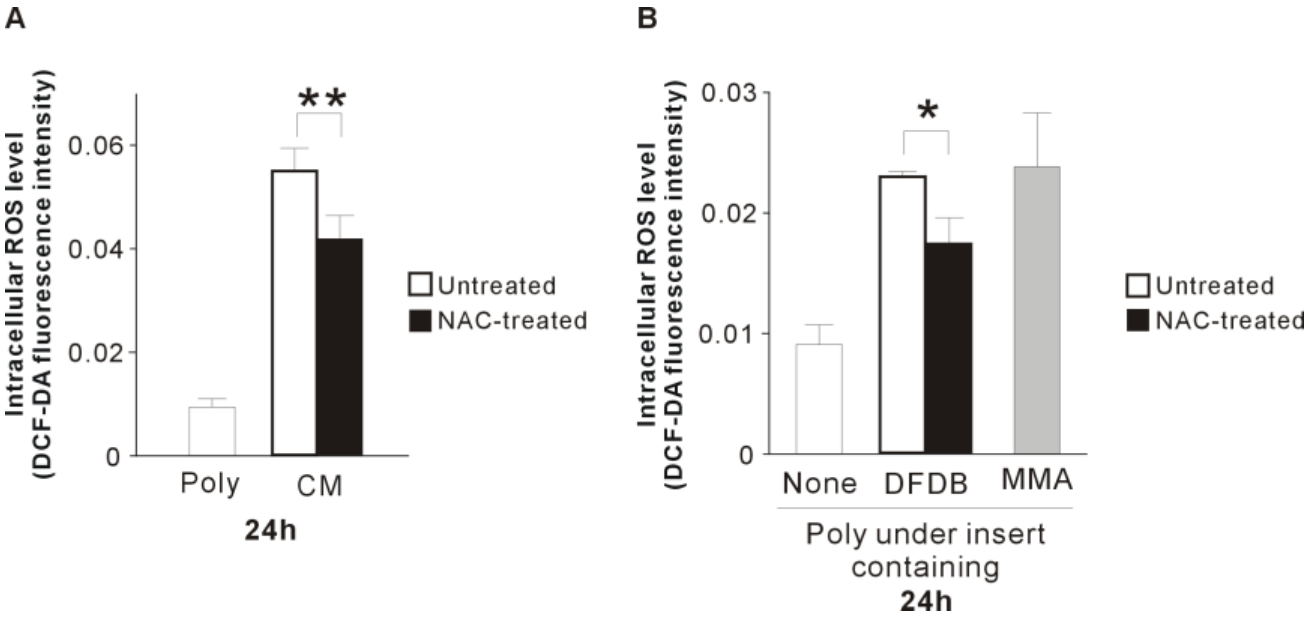
A
Intracellular ROS level
(DCF-DA fluorescence intensity)
0.06
0.04
0.02
0
**
Poly
CM
24h
Untreated
NAC-treated
B
Intracellular ROS level
(DCF-DA fluorescence intensity)
0.03
0.02
0.01
0
*
None
DFDB
MMA
Poly under insert
containing
24h
Untreated
NAC-treated

Figure. 4

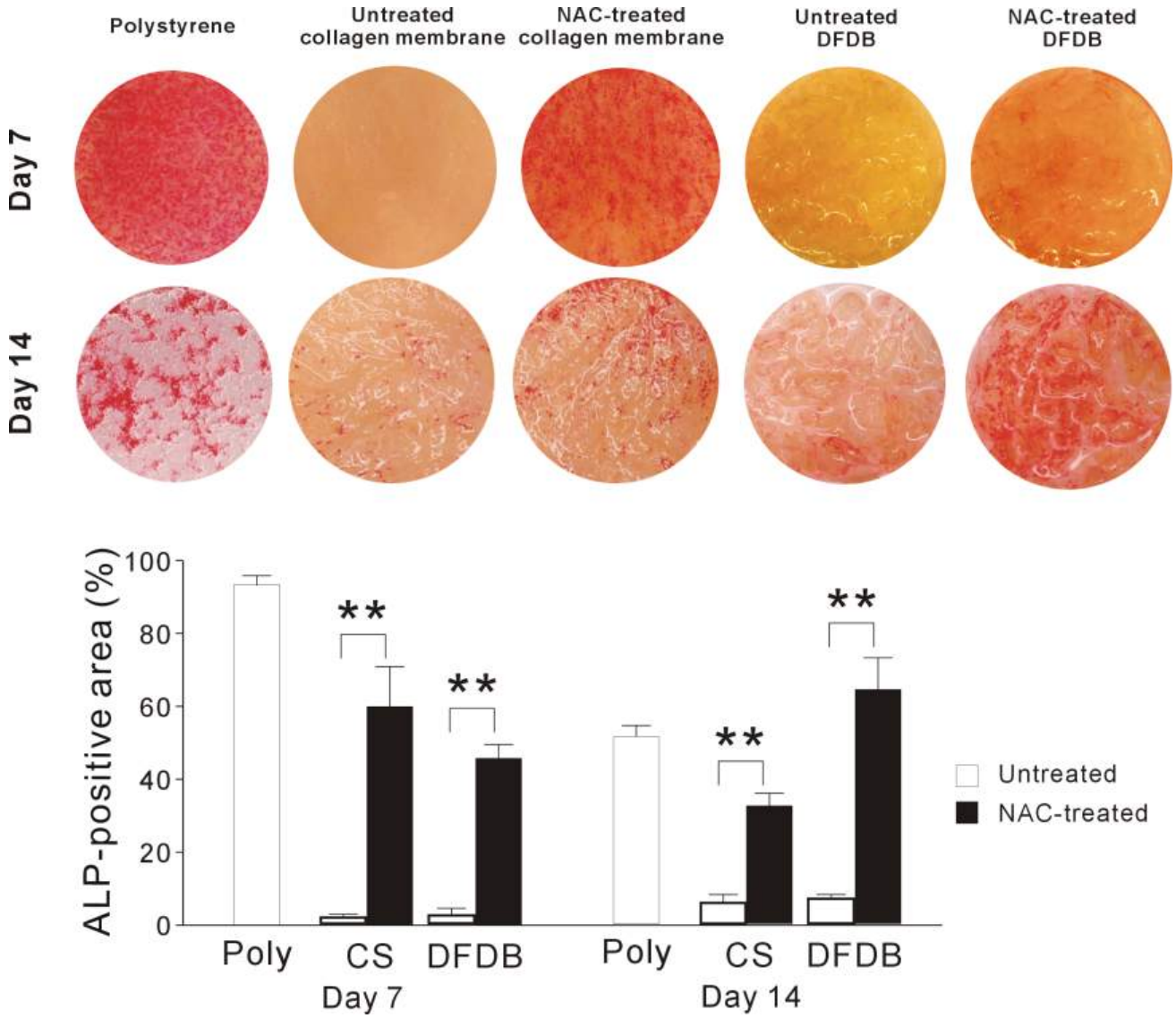
Polystyrene
Untreated collagen membrane
NAC-treated collagen membrane
Untreated DFDB
NAC-treated DFDB
Day 7
Day 14
ALP-positive area (%)
100
80
60
40
20
0
**
**
**
**
Poly
CS
DFDB
Poly
CS
DFDB
Day 7
Day 14
Untreated
NAC-treated

Figure. 5

Legends

Fig. 1. Images and schemas showing osteoblastic co-culture models with collagen membrane (CM) and demineralized freeze-dried bone particles (DFDB). (A) Co-culture model with physical contact between osteoblasts and CM or DFDB, where cells were directly seeded onto materials and (B) Co-culture model without physical contact between osteoblasts and DFDB, where cells were seeded onto polystyrene underlying culture insert containing material with 0.04-μm pore bottom suspended at 3.0 mm distance from polystyrene substrate.

Fig. 2. (A) Results of cell viability and apoptosis analysis using flow cytometry with dual staining for annexin V and propidium iodide (PI) in osteoblastic cultures grown on collagen membrane (CM) with or without N-acetyl cysteine (NAC) pretreatment or on polystyrene (Poly) at 24 hr after seeding. Flow cytometric images are shown on the top, and the percentages of viable cells (quadrant 3 in the top images) and dead cells (quadrants 1, 2 and 4) are shown on the bottom. Data are mean ± SD (N = 3). $^{**}p < 0.01$, (Bonferroni correction). (B) Results of attached viable cell number quantified by Calcein fluorophotometry in osteoblastic cultures on collagen membrane (CM) with or without N-acetyl cysteine (NAC) pretreatment or on polystyrene (Poly) at 24 hr after seeding. Data represent mean ± SD (N = 3). $^{**}p < 0.01$ (Bonferroni correction).

Fig. 3. Results of attached viable cell number quantified by Calcein fluorophotometry in osteoblastic cultures on demineralized freeze-dried bone particle (DFDB) with or without N-acetyl cysteine (NAC) pretreatment or on polystyrene (Poly) (A) or on polystyrene under a submicron porous-bottomed culture insert containing none, methyl methacrylate monomer (MMA) or DFDB with or without NAC pretreatment (B) at 24 hr after seeding. Data represent mean ± SD (N = 3). $^{**}p < 0.01$, $^{*}p < 0.05$ (Bonferroni correction).

Fig. 4. Results of intracellular ROS level evaluated by DCF-DA fluorophotometry of osteoblasts

cultured on collagen membrane (CM) with or without N-acetyl cysteine (NAC) pretreatment or on polystyrene (Poly) (A) or on polystyrene under a submicron porous-bottomed culture insert containing none, methyl methacrylate monomer (MMA) or demineralized freeze-dried bone particles (DFDB) with or without NAC pretreatment (B) at 24 hr after seeding. Data represent mean ± SD (N = 3). $^{**}p < 0.01$, $^{*}p < 0.05$ (Bonferroni correction).

Fig. 5. Results of alkaline phosphatase (ALP) staining on days 7 and 14 of osteoblastic cultures on collagen membrane (CM) or demineralized freeze-dried bone particles (DFDB) with or without N-acetyl cysteine (NAC) pretreatment or on polystyrene. Top panels show representative images of ALP-stained culture. Bottom histogram shows percentage of ALP-positive area to overall culture area measured using digital image analyzer. Data represent mean ± SD (N = 3). $^{**}p < 0.01$ (Bonferroni correction).