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N-acetyl Cysteine Alleviates Cytotoxicity of Bone Substitute

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ABSTRACT

Lack of cytocompatibility in bone substitutes impairs healing in surrounding bone. Adverse biological events around biomaterials may be associated with oxidative stress. We hypothesized that a clinically used inorganic bone substitute is cytotoxic to osteoblasts due to oxidative stress and that N-acetyl cysteine (NAC), an antioxidant amino acid derivative, would detoxify such material. Only 20% of rat calvaria osteoblasts were viable when cultured on commercial deproteinized bovine bone particles for 24 hr, whereas this percentage doubled on bone substitute containing NAC. Intracellular ROS levels markedly increased on and under bone substitutes, which were reduced by prior addition of NAC to materials. NAC restored suppressed alkaline phosphatase activity in the bone substitute. Proinflammatory cytokine levels from human osteoblasts on the bone substitute decreased by one-third or more with addition of NAC. NAC alleviated cytotoxicity of the bone substitute to osteoblastic viability and function, implying enhanced bone regeneration around NAC-treated inorganic biomaterials.

KEY WORDS: apoptosis, reactive oxygen species (ROS), anti-oxidant, bone regeneration, inorganic biomaterial.

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N-acetyl Cysteine Alleviates Cytotoxicity of Bone Substitute

INTRODUCTION

An inorganic bone substitute as represented by deproteinized bovine bone particles is frequently used in preprosthetic alveolar ridge augmentation. Bone substitute is placed directly within the bone cavity or on the surface of a decorticated osseous ridge to serve as a three-dimensional solid scaffold for osteogenic cell-induced bone formation (Somanathan and Simunek, 2006). Osteocompatibility of the bone substitute is crucial to the success of bone augmentation, and an adverse osteoblastic response may result in impairment of bone formation and prolongation of healing time. Although there are concerns regarding the cytocompatibility of inorganic materials (Ignjatovic *et al.*, 2006; Liu *et al.*, 2008), the compatibility of commercially available inorganic bone substitutes with osteoblasts remains to be clarified.

Recently, certain types of artificial devices/materials have been reported to exert an adverse biological impact on cells. Expanded polytetrafluoroethylene (e-PTFE) induced neutrophil death (Kaplan *et al.*, 1994; Nadzam *et al.*, 2000; Patel *et al.*, 2007), and reduced mitochondrial activity in both periodontal ligament fibroblasts and in an osteosarcoma cell line (Alpar *et al.*, 2000) by contact stimuli. Titanium alloy activated monocytes and macrophages, resulting in damage to co-cultured osteoblasts (Tsaryk *et al.*, 2007). Octacalcium phosphate induced nitric oxide production in cultured articular chondrocytes, possibly leading to degenerative arthropathies (Carter *et al.*, 2002). Inflammatory cytokine production in osteoblasts was increased in the presence of hydroxylapatite particles (Lenz *et al.*, 2009). Although the underlying pathological mechanism remains to be elucidated, an association has been suggested between the adverse biological effects induced by biomaterials and excessive generation of intracellular reactive oxygen species (ROS), which results in oxidative stress on cells (Luo *et al.*, 1998; Tsaryk *et al.*, 2007; Keegan *et al.*, 2008).

N-acetyl cysteine (NAC) is an antioxidant amino acid derivative, and a sulfhydryl group, the functional moiety of NAC, directly neutralizes ROS (Schweickl *et al.*, 2006; Spagnuolo *et al.*, 2006). NAC can be incorporated into a cell and deacetylated into L-cysteine, a precursor of glutathione (Zafarullah *et al.*, 2003; Schweickl *et al.*, 2006), which plays a central role in intracellular redox balance (Taylor *et al.*, 2002). This antioxidant capacity can protect cells from oxidative stress by directly scavenging extracellular ROS and compensating for the depletion of intracellular glutathione levels (Gillissen *et al.*, 1997; Gillissen and Nowak, 1998). In fact, NAC prevented the suppression of cell viability and function in fibroblasts and dental pulp cells caused by resin (Att *et al.*, 2009; Yamada *et al.*, 2009), which exhibits cytotoxicity *via* oxidative stress on cells (Kojima *et al.*, 2008). We hypothesized that NAC would

detoxify inorganic bone material if such material is cytotoxic to osteoblasts due to oxidative stress. The objectives of this study were to (1) determine whether commercial inorganic bone substitute negatively affects the viability, behavior, and function of osteoblasts in association with oxidative stress, and, if so, (2) explore the mechanisms underlying that cytotoxicity, and (3) determine whether NAC detoxifies that material and prevents osteoblast death and dysfunction.

MATERIALS & METHODS

Bone Substitute and NAC Preparation

A 0.04-g quantity of deproteinized bovine cancellous bone particles with a diameter of 0.25-1.0 mm (Bio-oss[®] Cancellous, Osteohealth, Shirley, NY, USA) was placed in each well on a culture-grade polystyrene 12-well plate. We prepared a NAC stock solution by dissolving NAC powder (Sigma-Aldrich, St. Louis, MO, USA) in HEPES buffer (1 mol/L stock, pH 7.2) which had been shown not to influence osteoblast viability (Yamada and Ogawa, 2009). We prepared the NAC-treatment solution by mixing the NAC stock solution with alpha-modified Eagle's medium (α -MEM, Gibco BRL Div. of Invitrogen, Gaithersburg, MD, USA) to a volume ratio of 1:49. Immediately before cell seeding, a 250- μ L quantity of α -MEM or NAC-treatment solution was added to the bone substitute.

Rat Osteoblastic Culture

Calvarial osteoblasts isolated from 8-week-old male Sprague-Dawley rats were grown with osteoblastic media as described in the Appendix. The cells were seeded onto polystyrene, untreated bone substitute, or NAC-treated bone substitute at a density of 3×10^4 cells/cm² in 1.0 mL osteoblastic media without ascorbic acid. A polystyrene culture containing 5 mM NAC was also prepared for evaluation of NAC cytocompatibility. The medium was renewed every 3 days.

To evaluate the biological effect of bone substitute on osteoblasts under non-physical contact conditions, we used a non-contact co-incubation model using a culture insert chamber with a translucent bottom (0.04- μ m pore filter) (Millicell, Millipore, Bedford, MA, USA). The chamber, containing 0.04 g bone substitute and 250 μ L α -MEM or NAC-treatment solution, was suspended above a polystyrene substrate on which rat osteoblasts were seeded to a density of 3×10^4 cells/cm² 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 culture. These study protocols were approved by the University of California Los Angeles Chancellor's Animal Research Committee.

Cell Viability, Intracellular ROS, Glutathione Level, and Osteoblastic Function Analysis

Annexin-V-based flow cytometric cell viability and apoptosis evaluation, attached cell count, intracellular ROS quantification, and confocal laser microscopic analysis of cell morphology and intracellular ROS localization were performed in rat osteoblast cell

culture on polystyrene, bone substitute, and on polystyrene under bone substitute at 24 hrs after seeding. Osteoblast functional activity on polystyrene and on bone substitute was assessed with alkaline phosphatase (ALP) staining at day 7. Total intracellular glutathione level was quantified in osteoblast polystyrene culture added with or without NAC at 24 hrs after seeding. Methodologies of all assays are described in the Appendix.

Evaluation of Inflammatory Responses of Osteoblasts on Bone Substitute

Inflammatory cytokine [interleukin (IL)-1 β , IL-2, IL-4, IL-6, and IL-8] production of human bone-marrow-derived mesenchymal stem cells on polystyrene, the untreated, or the NAC-added bone substitute at day 2 was assessed by means of the multiplex cytokine array system. Methodological details are described in the Appendix.

Statistical Analysis

All experiments were repeated 3 times, except for the cell morphology experiment in which 6 samples were evaluated. All repeated measurement results were expressed as means \pm SD, and significant differences ($p < 0.05$) among the experimental groups were evaluated with a one-way ANOVA or Student's *t* test. When appropriate, the Student-Newman-Keuls multiple comparisons test (SNK test) was used *post hoc*.

RESULTS

NAC Increases Viability of Osteoblasts on Bone Substitute

Flow cytometric analysis revealed that only 20% of osteoblasts survived on deproteinized bovine bone, in contrast to 90% on polystyrene, at 24 hrs after seeding ($p < 0.01$, SNK test) (Fig. 1A and Appendix Fig. 1). Cell death on bone substitute was characterized by apoptosis. Suppressed osteoblast viability on the substitute was ameliorated by pre-treatment of the material with NAC. Percentage of viable cells on the material increased two-fold by the pre-addition of NAC ($p < 0.01$). NAC reduced the percentage of early apoptotic cells on the bone substitute from 45% to 26%. Addition of NAC showed no effect on viability or apoptotic appearance of osteoblasts on polystyrene ($p > 0.05$).

NAC Ameliorates Impaired Osteoblast Adhesion and Marked Intracellular ROS Generation on Bone Substitute and Increases Cellular Glutathione Levels

Confocal laser microscopy revealed largely expanded osteoblasts developing a cytoskeleton and cellular processes with little ROS detection on polystyrene at 24 hrs after seeding, whereas attached osteoblasts on bone substitute were round, small, and filled with intracellular ROS (Appendix Fig. 2). Even more largely expanded osteoblasts and lower ROS production were observed on NAC-treated bone substitute than on untreated material. Area, perimeter, and Feret's diameter of osteoblasts on

untreated bovine mineral were only 15% or less of those on polystyrene ($p < 0.01$) (Fig. 1B). However, pre-addition of NAC yielded a greater than three-fold increase in values for those parameters compared with those for untreated material. The number of attached cells on bone substitute was less than one-tenth of the number on polystyrene at 24 hrs after seeding ($p < 0.01$) (Fig. 1C). NAC increased attached cell number on bone substitute by 160% compared with that on untreated substitutes ($p < 0.01$). Intracellular ROS levels in osteoblasts exhibited a 16-fold increase on untreated bovine mineral compared with that on polystyrene ($p < 0.01$) (Fig. 1D), and decreased to less than half of that on untreated materials on NAC-added substitute ($p < 0.01$). Numbers of attached cells, cell morphology, and intracellular ROS levels on polystyrene at 24 hrs after seeding were not affected by NAC (Figs. 1B-1D). NAC-addition resulted in a 70% increase in cellular glutathione levels of osteoblastic polystyrene culture at 24 hrs after seeding ($p < 0.01$, Student's *t* test) (Fig. 1E).

NAC Restores Suppressed ALP Activity on Bone Substitute

On day 7, ALP activity was hardly detected in cells on bovine mineral, in contrast to extended and intensive positive reaction for ALP staining on polystyrene (Fig. 2 and Appendix Fig. 3). The percentage of ALP-positive area was 0.05% on bone substitute, whereas it was 80% on polystyrene. However, ALP activity on bone substitute was substantially elevated by the pre-addition of NAC. The percentage of the ALP-positive area on bone substitute increased up to 50% by NAC pre-treatment ($p < 0.01$).

NAC Reduces Pro-inflammatory Cytokine Production in Osteoblasts on Bone Substitute

Cytokine multiplex analysis revealed that human osteoblasts on bone substitute secreted a 4- to 12-times greater amount of

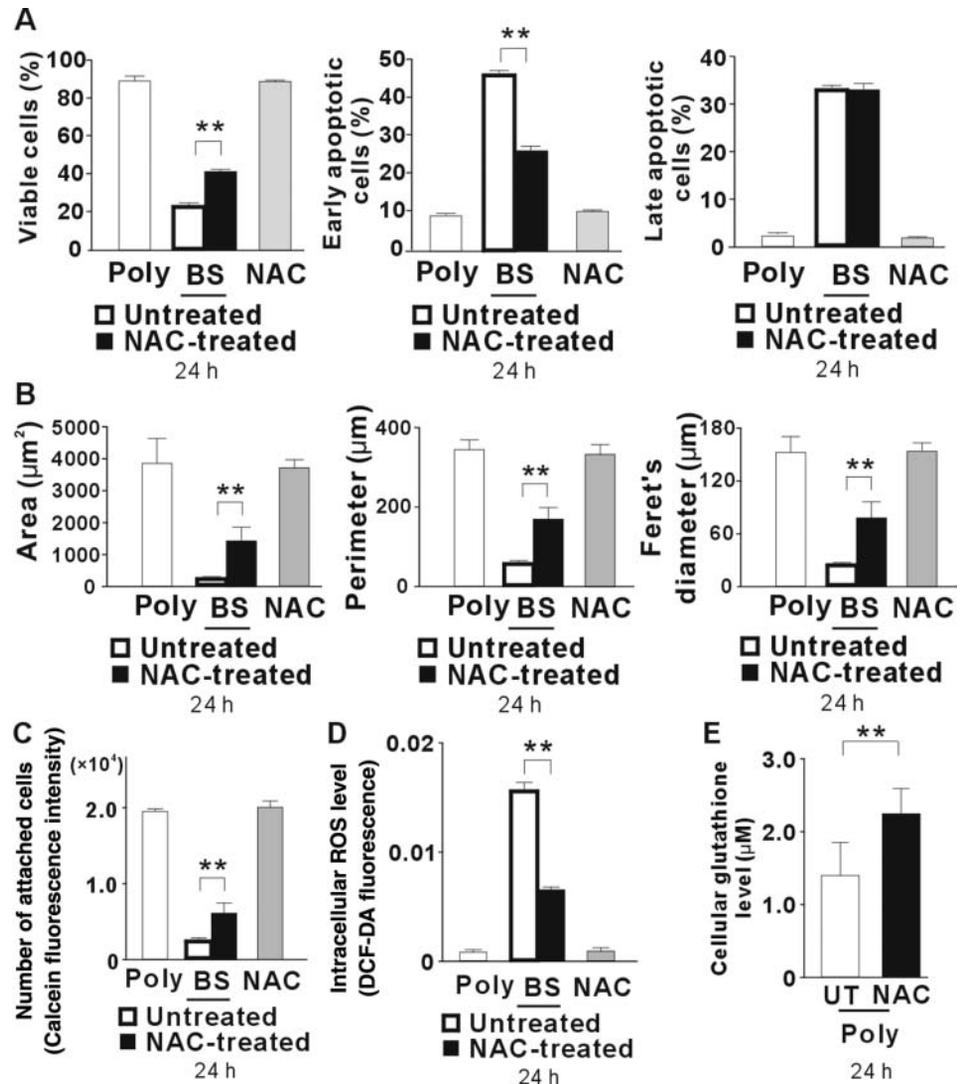


Figure 1. Cell viability and apoptosis analysis by Annexin V-based flow cytometry in osteoblasts cultured on polystyrene with or without NAC and on deproteinized bovine bone particles with or without NAC at 24 hrs after seeding (A). Percentages of viable cells (quadrant 3 in upper images), early apoptotic cells (quadrant 4), and late-apoptotic cells (quadrant 2) are shown. Results of cell morphometric measurement (B), attached cell number quantified by Calcein fluorophotometry (C), and intracellular ROS level evaluated by DCF-DA fluorophotometry (D) in cultures on polystyrene or on bone substitute with or without NAC at 24 hrs after seeding. Poly, polystyrene culture; BS, culture on bone substitute with or without NAC; NAC, polystyrene culture with NAC. Total glutathione quantification in osteoblastic polystyrene culture added with or without 5.0 mmol/L NAC at 24 hrs after seeding (E). UT, polystyrene culture without NAC; NAC, polystyrene culture with 5.0 mmol/L NAC. Data are mean \pm SD [N = 3 (A, C, D, E); N = 6 (B)]. ** $p < 0.01$ (SNK-test).

pro-inflammatory cytokines such as IL-1 β , IL-2, IL-4, IL-6, and IL-8 than those on polystyrene at day 2 ($p < 0.01$) (Fig. 3). Pre-addition of NAC to bone substitute reduced cytokine production in human osteoblasts on bone substitute by 40-60% ($p < 0.01$).

Harmful Effect of Bone Substitute on Osteoblasts under Non-contact Conditions Alleviated by NAC

Ninety-six percent of osteoblasts cultured on polystyrene were viable at 24 hrs after seeding. This percentage decreased to 88% by non-contact co-incubation with bone substitute ($p < 0.01$)

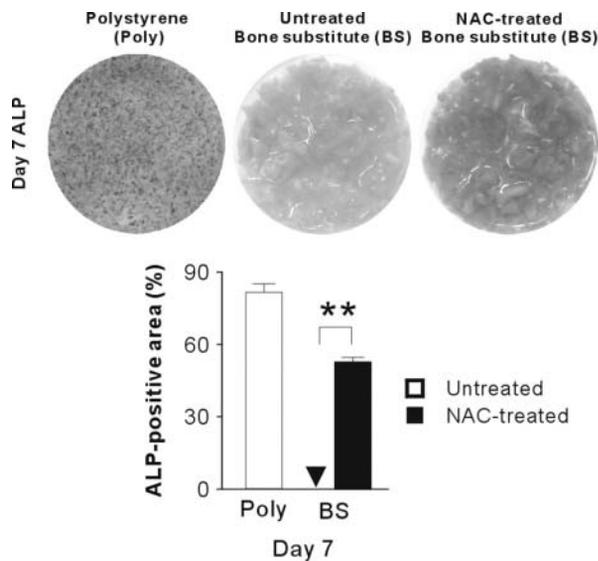


Figure 2. Alkaline phosphatase (ALP) staining of osteoblast cultures on polystyrene and on deproteinized bovine bone particles with or without NAC at day 7. Top panels show representative images of ALP-stained cultures. Bottom histogram shows percentage of ALP-positive area to overall culture area measured by means of a digital image analyzer. Data are mean \pm SD (N = 3). Poly, polystyrene culture; BS, culture on bone substitute with or without NAC; NAC, polystyrene culture with NAC. ** $p < 0.01$ (SNK-test).

(Fig. 4A). However, this percentage increased to 92% or more by the pre-addition of NAC to the material. Bone substitute induced a 1.5-times and 3.5-times increase in the percentage of early and late apoptosis, respectively, in underlying osteoblastic culture on polystyrene compared with that on polystyrene without co-incubation with bone substitute (2.7% and 1.1%, respectively) ($p < 0.01$). NAC significantly reduced the percentage of apoptosis on the polystyrene culture underneath the bone substitute. At 24 hrs after seeding, the number of attached cells on polystyrene under the bone substitute decreased to half of that on polystyrene without co-incubation. However, this number increased to a level equivalent to that on polystyrene without co-incubation by pretreatment of the material with NAC (Fig. 4B). The intracellular ROS level was elevated on polystyrene underneath the bone substitute by 1.9 times that on polystyrene without co-incubation at 24 hrs after seeding ($p < 0.01$) (Fig. 4C). The NAC-treated bone substitute significantly prevented ROS elevation in underlying osteoblasts on polystyrene ($p < 0.01$).

DISCUSSION

Apoptosis, inflammatory response, and dysfunction in osteoblasts cultured on bovine bone-derived substitute were accompanied by marked intracellular ROS generation in the present study. Physicochemical stimuli such as ultraviolet light, ionizing radiation, chemical compounds, and extracellular ROS can yield an extraordinary increase in intracellular ROS generation (Schweikl *et al.*, 2006). Excess intracellular ROS depletes cellular glutathione and disturbs the cellular redox balance, resulting in

oxidative stress (Feinendegen, 2002). Stressed cells undergo oxidative challenge such as lipid peroxidation and DNA strand breaks, with dysregulation of cellular survival or differentiation-related signaling pathways (Feinendegen, 2002; Schweikl *et al.*, 2006), leading to induction of apoptosis and cellular dysfunction (Yamada *et al.*, 2008; Att *et al.*, 2009; Tsukimura *et al.*, 2009).

Bone substitute has the inherent potential to induce oxidative stress. Generally, organ-derived bone substitutes undergo some type of physicochemical treatment during manufacture, including deproteination and inactivation of pathogens (Tadic and Epple, 2004), which results in alteration of surface properties and the addition of chemical remnants to the material (Carter *et al.*, 2002; Bertazzo and Bertran, 2008). This implies that xenogenetic bone replacement material may biologically influence cells not only by contact stimuli, but also by release of certain chemical agents accompanying the material's surface dissolution (Accorsi-Mendonca *et al.*, 2008). In this study, osteoblast adverse responses were seen when osteoblasts were directly seeded onto the deproteinized bovine bone substitute and on underlying polystyrene under non-contact conditions. It will be of great interest for future research to identify oxidative stress-inducing factors in inorganic bone substitute, which are suspected to include the negative influence of surface properties on cell adhesion (Dent *et al.*, 2003), the elution of chemical substances, and ROS generation (Stefaniak *et al.*, 2009) originating in the dissolution of materials.

N-acetyl cysteine reduced the adverse biological effects of bone substitute on osteoblasts in the present study. Its ability to detoxify oxidative stress-evoking toxic material has been well-proven. Suppressed viability and function in extracellular matrix-producing cells on polymethyl methacrylate (PMMA)-based resin were recovered by the addition of NAC to the resin (Att *et al.*, 2009; Yamada *et al.*, 2009). Mechanisms underlying the detoxification of biomaterial by NAC are thought to be wide-ranging. NAC can directly scavenge ROS and toxic compounds with its functional moiety (Schweikl *et al.*, 2006; Yamada and Ogawa, 2009). In addition, NAC has the cellular pharmacodynamics to elevate intracellular glutathione levels, as shown in the present study, which indicated that NAC may enhance the anti-oxidant resistance of osteoblasts against oxidative stress from materials (Tsukimura *et al.*, 2009). Furthermore, NAC might modulate cysteine-containing transcriptional factors such as nuclear factor kappa B, which regulates cell survival and differentiation (Paranjpe *et al.*, 2007).

A variety of immune and tissue-forming cells produces pro-inflammatory cytokines. Some type of biomaterial may increase osteoblastic pro-inflammatory cytokine production, especially IL-6 and IL-8 (Huang *et al.*, 2005; Lenz *et al.*, 2009). IL-6 acts as a multiple major mediator of pro-inflammatory processes such as B-cell differentiation (Ishimi *et al.*, 1990). IL-8 is a chemokine to induce neutrophil chemotaxis and activation (Baggiolini *et al.*, 1994). Dysregulated generation of these cytokines in bone tissue may lead to local inflammatory development, increased osteoclastic bone resorption, and reduced bone formation (Vermees *et al.*, 2001). Moreover, these pro-inflammatory cytokines are involved in cross-talk between osteoblasts and immune cells

(Rauner *et al.*, 2007). It will be of great interest for further research to examine the influences of bone substitute and the effect of NAC on the osteoimmunological network.

Biological amelioration of bone substitute may lead to the upgrading of surrounding bone regeneration, *i.e.*, acceleration of bone formation and enhancement of bone volume and bone-material integration strength. Indeed, NAC detoxified PMMA-based bone cement, resulting in remarkably enhanced bone-contact area, surrounding bone volume, and biomechanical strength at the bone-material interface as compared with the original cement alone (Tsukimura *et al.*, 2009). NAC-induced improvement in the biocompatibility of bone substitute may functionalize the material and offers potential in the development of new breakthrough bone substitutes.

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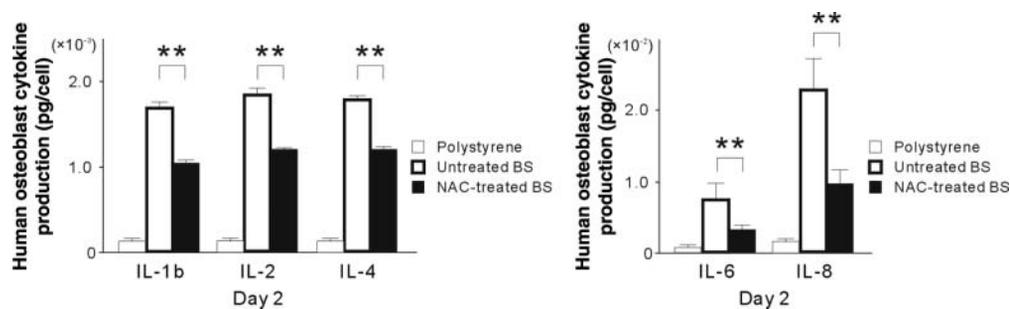


Figure 3. Inflammatory cytokine production in human osteoblasts cultured on polystyrene or on deproteinized bovine bone particles with or without NAC at day 2. Results of IL-1, IL-2, and IL-4 are shown on the left of the histogram and those of IL-6 and IL-8 on the right. Data are mean ± SD (N = 3). Poly, polystyrene culture; BS, culture on bone substitute with or without NAC. **p < 0.01 (SNK-test).

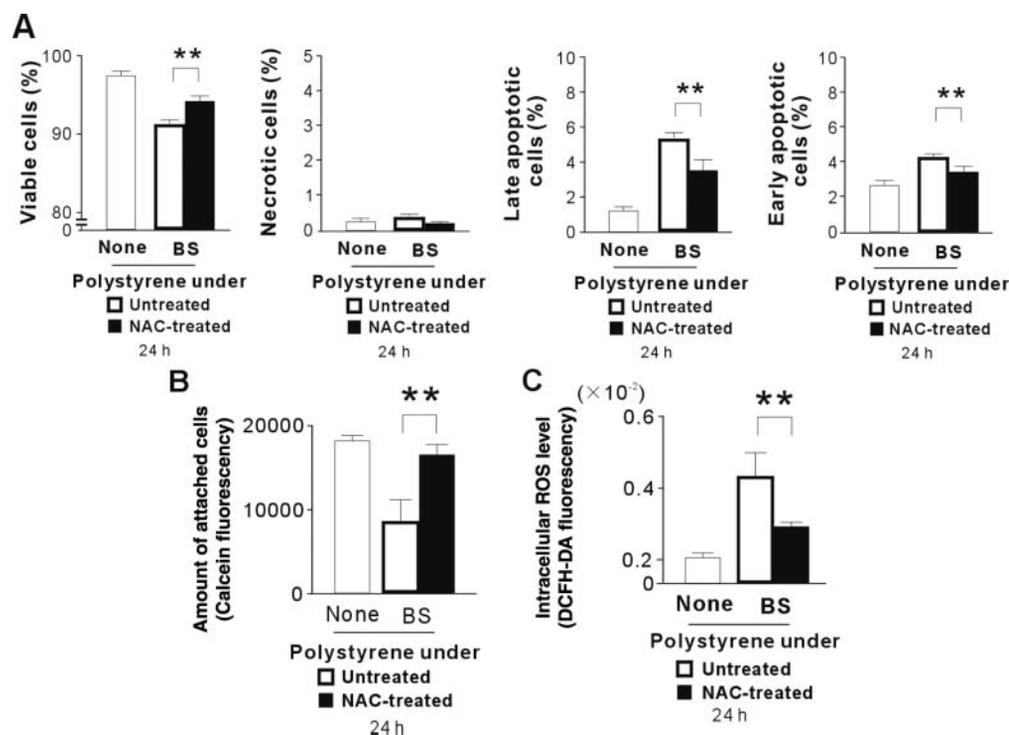


Figure 4. Cell viability, the number of attached cell and intracellular ROS level on polystyrene with or without co-cubation with deproteinized bovine bone particle under non-contact condition. (A) Cell viability and apoptosis analysis by Annexin V-based flow cytometry in osteoblasts cultured on polystyrene under deproteinized bovine bone particles with or without NAC at 24 hrs after seeding. Flow cytometric images are shown at the top, and percentages of viable cells (quadrant 3 in upper images), necrotic cells (quadrant 1), early apoptotic cells (quadrant 4), and late apoptotic cells (quadrant 2) are shown at the bottom. (B) Results of attached cell numbers quantified by calcein fluorophotometry in osteoblasts cultured on polystyrene under bone substitute with or without NAC at 24 hrs after seeding. (C) Intracellular ROS level evaluated by DCF-DA fluorophotometry in osteoblasts cultured on polystyrene under bone substitute with or without NAC at 24 hrs after seeding. Data are mean ± SD (N = 3). Poly, polystyrene culture; BS, culture on bone substitute with or without NAC. **p < 0.01 (SNK-test).

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