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N-acetyl cysteine improves affinity of beta-tricalcium phosphate granules for cultured osteoblast-like cells

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Short title: Upgraded osteocompatibility of β-TCP by NAC

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Abstract
Enhancement of bone substitute’s biocompatibility may accelerate healing of surrounding bone. Although widely used as a biodegradable alloplastic bone substitute for alveolar bone augmentation, the osteocompatibility of beta-tricalcium phosphate (β-TCP) remains to be proven. The adverse cellular response to biomaterials is associated with oxidative stress. We hypothesized that commercially available β-TCP granules for clinical use, caused oxidative stress and was not optimal in osteocompatibility and that application of antioxidant amino acid derivative N-acetyl cysteine (NAC) would improve osteoblastic responses to the material. Only 20% of rat calvarial osteoblasts cultured on β-TCP granules remained viable at 24 hr after seeding as opposed to 90% on polystyrene. Cell death on β-TCP granules was characterized by necrosis. However, the percentage of viable osteoblasts cultured on β-TCP granules showed a 100% increase with pre-treatment with NAC. NAC restored suppressed alkaline phosphatase activity on β-TCP granules at day 5. Intracellular ROS level on β-TCP granules was 16-fold greater than that on polystyrene, but decreased by half with pre-treatment with NAC. Cell death and intracellular ROS elevation were also induced in polystyrene culture under β-TCP granules even when the osteoblasts were not in direct contact with the β-TCP granules. NAC, however, prevented induction of cell death and elevation of intracellular ROS under β-TCP granules. These results indicate that commercially available β-TCP granules negatively affect cultured osteoblastic viability and function via oxidative stress and that NAC improves these negative responses to the material. This implies enhanced bone regeneration around biodegradable calcium phosphate-based bone substitute by NAC.

Keywords: reactive oxygen species (ROS); antioxidant; bone substitute; biocompatibility; bone regeneration
Beta-tricalcium phosphate (β-TCP) granules is a crystal form of tricalcium phosphate (TCP) \([\text{Ca}_3(\text{PO}_4)_2]\) which is categorized as calcium orthophosphate subfamily among calcium phosphate (CP) compounds. Its chemistry is characterized by higher water solubility than stoichiometric synthetic hydroxyapatite (HA), which results in dissolution and absorption \textit{in vivo} [1-4]. Moreover, its physical strength is against a pressure of soft tissue covering over the implantation site. Its slow biodegradation property harmonizes with bone formation and remodeling process and results in a displacement of the material to bone tissue [2, 3]. These β-TCP’s characteristics allow it to serve as a 3-dimensional scaffold for bone regeneration [5]. Therefore, β-TCP is used as a biodegradable alloplastic bone substitute for alveolar ridge and sinus floor augmentation for implant therapy [6, 7].

The current definition of biocompatibility is given as the biological properties of a material not to interfere with cellular response and subsequent tissue formation [8]. 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 [9]. Improvement of osteoblastic cellular responses to biomaterial results in enhancement of regenerative outcomes in the volume, quality and forming period of new bone on/around the material [10]. Generally, commercially available β-TCP products are supplied as highly purified granules, and are placed directly onto a decorticated osseous ridge or into a bone cavity. Therefore, compatibility of this material to osteoblasts is crucial for the successful clinical outcome.

Cytocompatibility of biomaterial is generally assessed by a culture study [11]. There is the discrepancy in opinion about the cytocompatibility of CP compounds between culture and animal studies. It has been generally recognized that biodegradable CP materials shows less biological negative impact in an \textit{in vivo} experiment [2, 12]. By contrast, previous culture studies about the cytocompatibility of CP material were contradictory [13-19]. According to one explanation, an
enclosed environment such as in vitro culture experiment allows biodegradable CP compounds to exert harmful effects on cells by physicochemical stimuli during the dissolution, whereas the in vivo environment could sufficiently transport the toxic products from the material away by regional circulation during the initial phases of wound healing [12]. This means that the bone formation around CP compound requires a resistance of cell and tissue against the material’s biological adverse effects and that the materials are not optimal in their cytocompatibility. In addition, regional circulation at the implant site is not always adequate. Therefore, it is required to investigate the osteoblastic affinity to commercially available β-TCP granules under an enclosed environmental.

A number of recent studies have reported that well-known artificial biomaterials exerted a cytotoxic effect on cells. Expanded polytetrafluoroethylene (e-PTFE) killed neutrophils by contact stimuli [20-22]. Mitochondrial metabolic activity in periodontal ligament fibroblasts and osteosarcoma cell line showed a reduction on e-PTFE [23]. Titanium alloy stimulated immunocompetent cells such as monocytes and macrophages, resulting in injury of co-cultured osteoblasts [24]. Calcium carbonate increased proinflammatory cytokine production of peripheral human leukocytes [25]. Zirconium oxide, titanium alloy and hydroxylapatite debris from orthopedic implants evoked inflammatory reactions in human osteoblasts [16]. Deproteinized bovine bone mineral particles induced cell death, loss of function and an increase of proinflammatory cytokine production in cultured osteoblasts [26]. Recently, oxidative stress caused by excessive generation of intracellular reactive oxygen species (ROS) has been suggested to be associated with the adverse biological effects of such materials [24, 26-29].

N-acetyl cysteine (NAC) is an anti-oxidant amino acid derivative. The functional base of NAC, its sulphhydryly group, can directly eliminates ROS [30]. NAC can membrane-permeably be ingested into a cell and deacetylated into L-cysteine which is a precursor of glutathione [31, 32]. Glutathione serves as an essential molecule for intracellular redox balance and detoxification systems [33].
Therefore, NAC prevents oxidative stress on cells by scavenging extrinsic ROS and compensating for depleted intracellular glutathione levels [34, 35]. NAC rescued osteoblasts, dental pulp cells and fibroblasts from polymethyl methacrylate-based bone cement- and dental resin-induced loss of cell viability and dysfunction due to oxidative stress-mediated cytotoxicity [10, 36-38]. Moreover, NAC could alleviate cell death and dysfunction of osteoblasts cultured on bovine bone-derived bone substitute [26]. The purpose of this in vitro study was to determine 1) whether commercially available β-TCP bone substitute exerts a negative biological influence on osteoblasts in association with oxidative stress and, if so, 2) whether impairment of osteoblastic viability and function on β-TCP granules was prevented by application of NAC.

Materials and methods

Bone substitute and NAC preparation

A total of approximately 0.04 g β-TCP granular bone substitute (1.0-3.0 mm in diameter and 53-67% porosity) (OSferion® 60G2-5, Olympus Terumo Biomaterials Corp., Tokyo, Japan), was placed on the bottom of each well of 12-well culture-grade polystyrene plates. 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) to a volume ratio of 1:49. Immediately before cell seeding, the β-TCP granules were soaked with 250 μl α-MEM or NAC-treatment solution. NAC’s final concentration was 5mM, which was based upon the previous culture study [29].

Rat osteoblastic culture on and under bone substitute

Osteoblastic culture on β-TCP granules

Osteoblasts were derived from the parietal and 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 mm²) and digested with 0.25% collagenase (Sigma) for 12 hr. Liberated cells were collected and plated on 100-mm plastic tissue culture dishes with α-MEM supplemented with 15% fetal bovine serum, 50 μg/mL ascorbic acid, 10⁻⁸ M dexamethasone, 10 mM 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. Cells were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. At 80% confluence, the cells were detached using 0.25% trypsin-1 mM EDTA-4Na and seeded onto polystyrene substrates, untreated β-TCP granules or, NAC-treated β-TCP granules at a density of 3 × 10⁴ cells/cm² in 1.0 mL osteoblastic media without ascorbic acid. Cells were cultured for 5 days and 100 μl of fresh medium was added every 3 days, which simulated an enclosed biological environment with insufficient fluid circulation.

**Osteoblastic polystyrene culture under β-TCP granules**

To evaluate the biological effect of β-TCP granules on osteoblasts under non-physical contact conditions, a non-contact co-incubation model using a culture insert chamber with a translucent bottom (0.04-μm pore filter) (Millicell, Millipore, Bedford, MA, USA) was used. The chamber, containing 0.04 g β-TCP granules and 250 μl α-MEM or NAC-treatment solution, was suspended above a polystyrene substrate on which rat osteoblasts were seeded to a density of 3 × 10⁴ 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 (ARC #2005-175-12).

**Detection of cell viability and apoptosis**
Cell viability and apoptosis in the culture were evaluated on polystyrene and β-TCP granules and on polystyrene culture under the granules at 24 h after seeding by flow cytometry using an apoptosis detection kit ( Annexin V-FITC Kit, BD Bioscience, San Jose, CA, USA). Floating and attached cells were collected into a tube. After collecting the culture supernatant, polystyrene and β-TCP granular 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 FITC intensity (x-axis). 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. Scanning electron micrographs (SEM) of the selected cultures were obtained to confirm the absence of cell remnants on the substrates.

Alkaline phosphatase staining

On day 5, the cultures on polystyrene and β-TCP granules 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 stained images were analyzed for alkaline phosphatase (ALP)-positive areas, defined as [(stained area/total substrate area) × 100] (%), using an image analyzer (ImageJ, NIH, Bethesda, MD, USA).

Measurement of intracellular ROS level and number of attached cells

Amount of intracellular ROS production in attached cells on polystyrene and β-TCP granules and on polystyrene under the granules 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. At 24 hr after seeding, 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 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. Intensity was calibrated according to individual attached cell number as determined with a hematocytometer. Selected cultures were subjected to SEM to confirm the absence of cell remnants on the substrates.

**Statistical analysis**

All experiments were repeated 3 times. All repeated measurement results were expressed as means ± SD, and significant differences (p < 0.05) among the experimental groups were evaluated with a one-way ANOVA. When appropriate, the Bonferroni multiple comparison test was used for post hoc analysis.

**Results**

**NAC increases osteoblastic viability and reduces necrosis on β-TCP granules**

Flow cytometric analysis revealed that only 20% of osteoblasts were viable on β-TCP granules in contrast with 90% on polystyrene at 24 hr after seeding (p < 0.01, Bonferroni) (Fig. 1). Pretreatment with NAC solution increased the percentage of viable cells on β-TCP granules by 100% (p < 0.01). While necrosis was hardly detected on polystyrene (< 0.5%), 80% of osteoblasts on β-TCP granules were necrotic, which was reduced to 60% by NAC (p < 0.01).

**NAC restores suppressed ALP activity in osteoblasts on β-TCP granules**

At day 5, a positive reaction for ALP staining was barely detected on β-TCP granules, whereas polystyrene culture showed extended and intensive ALP activity (Fig. 2). The percentage of ALP-positive area was under 4% on β-TCP granules, whereas it was 80% on polystyrene. ALP
activity on β-TCP granules was remarkably enhanced by pretreatment with NAC. NAC increased the percentage of the ALP-positive area on β-TCP granules by up to 50% (p < 0.01).

**NAC increases attached cell number on β-TCP granules**

Untreated β-TCP granules showed a 75% reduction in the number of attached osteoblasts at 24 hr after seeding as compared to that on polystyrene (p < 0.01) (Fig. 3A). Pre-treatment with NAC resulted in a 120% increase in the number of attached cells on the material (p < 0.01).

**NAC alleviates marked intracellular ROS generation in osteoblasts on β-TCP granules**

Intracellular ROS level in osteoblasts at 24 hr after seeding elevated 16-fold on untreated β-TCP granules as compared to on polystyrene (p < 0.01) (Fig. 3B). However, such marked intracellular ROS generation on β-TCP granules was alleviated by pre-treatment with NAC, with NAC reducing intracellular ROS level to less than half of that on the untreated material (p < 0.01).

**NAC helps prevent osteoblastic death and intracellular ROS elevation under β-TCP granules**

The percentage of viable osteoblasts was 96% on polystyrene at 24 hr after seeding, which decreased to 88% under β-TCP granules (p < 0.01) (Fig. 4A). However, this percentage increased to 94% by pre-addition of NAC. Cell death in polystyrene culture under β-TCP granules was characterized as apoptosis. The percentage of early and late apoptosis increased by 2.7-times and 3.4-times, respectively, on polystyrene under β-TCP granules compared to that on polystyrene without superincumbent β-TCP granules (2.7% and 1.1%, respectively) (p < 0.01). These apoptotic incidents were significantly reduced by pre-addition of NAC (p < 0.01). The percentage of necrosis was significantly greater on polystyrene under β-TCP granules than on polystyrene without a co-incubation with β-TCP granules (p < 0.05), with NAC pre-treatment reducing levels to those on polystyrene without co-incubation (p > 0.05).
Intracellular ROS level showed a 2.4-fold increase on polystyrene under β-TCP granules over that on polystyrene without co-incubation with β-TCP granules at 24 hr after seeding (p < 0.01) (Fig. 4B). Addition of NAC to the β-TCP granules significantly prevented intracellular ROS generation in underlying osteoblasts on polystyrene (p < 0.01).

**Discussion**

Cellular/tissue response to CaP-based materials is a subject of controversy. Greater cell differentiation were observed on CaP-based materials than with polystyrene cultures [15, 17, 19]. It was advocated that no problems have been observed for the in vivo behavior of the CaP cement materials [12]. Contrastively, aseptic fibrous capsulation and local inflammation have been found in subcutaneous tissue implanted with CaP ceramics, including TCP [13]. Pure CaP bioceramics induced greater capsular thickness and more macrophages than aluminum-containing CaP compounds [39]. Several culture studies have reported adverse cellular responses to CaP-based materials. Ceramic discs fabricated with β-TCP markedly increased lactate dehydrogenase leakage levels, an indicator of cellular injury, in adipose-derived stem cells compared to control polystyrene [14]. Immunohistological study revealed that IL-1 beta, IL-2, IL-6 and TNF-infinity, and CD-4- and CD-14-positive cells were densely detected in fibrous capsules surrounding CaP materials [40], indicating an intensive inflammatory tissue response. Likewise, the present study demonstrated that β-TCP granules caused biological adverse events on osteoblasts under an enclosed environment. Culturing osteoblasts on the granules resulted in necrosis and suppression of ALP activity with extraordinary generation of intracellular ROS. Induction of osteoblast death and elevation of intracellular ROS level was seen even under non-physical contact conditions between cells and β-TCP granules. These results indicated that commercially available β-TCP granules have a potential to cause oxidative stress and a room to improve in the biological property.

Excluding certain types of bacteria, all organisms require oxygen for life. Once incorporated, this
oxygen transforms into ROS during the aerobic metabolic process. Physiologic ROS plays several biogenic roles, including an electron carrier, in enzymatic catalysis, as a neuromessenger, in oxidizing the attack molecules of the immunosystem for antigens and a cell-to-cell cross-talking agent [30, 41]. However, external stimuli such as bacterial infection, ultraviolet light, ionizing radiation, chemical compounds and extracellular ROS can induce excessive generation of intracellular ROS [30]. Excessive non-physiological intracellular ROS depletes cellular antioxidant molecules and causes oxidative stress on cells [42]. As a result, cells undergo lipid peroxidation, DNA strands break and dysregulation of cellular survival and differentiation-related signaling pathways occurs [30, 42], all inducing apoptosis and cellular dysfunction [36-38, 43].

The dissolution of bioabsorbable CP-based materials depends on the inherent chemical characteristics, and especially the solubility product constant, of the material [2, 44]. The chemical characteristics of β-TCP are well known. Generally, TCP, whose calcium (Ca)/phosphate (P) ratio is 1.5, has three crystal forms, α, β and γ, which are thermodynamically determined by the sintering temperature of amorphous TCP. Despite a lower solubility than that of the α-form, β-TCP is markedly more soluble than synthetic HA, which is confirmed by its solubility product constant (α-TCP: 3.16×10^{-26}; β-TCP: 2.19×10^{-30}; synthetic HA: 2.13×10^{-59}). This indicates that β-TCP releases Ca cations [Ca(2+)] and phosphate anion [P(i)], which play important roles on bone homeostasis, these with submergence in a liquid such as tissue fluid or culture media. Evidently, Ca(2+) modulates osteoblastic viability, motility, proliferation and differentiation through activation of calcium-sensing receptors and enhancement of Ca(2+) influx into cells and subsequent intracellular calcium signaling pathways [45, 46]. However, recent culture study indicated that Ca(2+) and P(i) can induce osteoblastic cell death in a coordinated manner [47-49]. Inorganic phosphate induced a profound loss of mitochondrial membrane potential of osteoblasts [47]. In addition, the presence of Ca (2+) helps P(i)-dependent death of cultured osteoblasts. The ion pair conspired at the level of the plasma membrane to induce intracellular changes that result in loss of
mitochondrial function, the subsequent cytosolic Ca(2+) elevation by the endoplasmic reticulum and the downstream events inducing apoptosis [49, 50]. More intriguingly, it was indicated that a cytotoxic responses of cells exposed to oxidative stress correlated with a sustained increase in cytosolic Ca(2+) concentration [51-53]. A association of oxidative stress with P(i)-induced cell death was also suggested in a certain type of cell [54]. In the present study, culturing osteoblasts on β-TCP granules resulted in an induction of cell death and reduced ALP activity with a marked increase of intracellular ROS level. Induction of cell death and increased intracellular ROS level in osteoblasts were observed even on polystyrene under co-culture condition with β-TCP granules, but without physical contact between the cells and the material. Although further pathological investigation is needed, an elevation of the local concentration of Ca(2+) and P(i) along with dissolution of TCP might be a key event in the mechanisms underlying the biological negative impact of β-TCP granules in the same way as other CaP-based material [12, 55]. Moreover, there are other possible mechanisms of how TCP granules induce adverse cellular responses and oxidative stress, such as micro-particle-related cellular damage [28, 56], the negative influence of surface properties on cell adhesion [57] and dissolution-induced extracellular ROS generation [58]. It will be of great interest for future research to demonstrate the underlying mechanism of negative osteoblastic cellular responses to β-TCP and other bioabsorbable CaP-based materials.

Application of NAC significantly alleviated cytotoxicity of β-TCP granules in the present study. A number of studies have indicated that NAC detoxifies oxidative stress-inducing cytotoxic materials. NAC restored suppressed viability and function in rat dental pulp cells and mucosal fibroblasts on polymethyl methacrylate (PMMA)-based dental resin [36-38]. Incorporation of NAC into PMMA-based bone cement prevented intracellular redox imbalance, apoptotic change and malfunction of cultured osteoblasts. The detoxification mechanism of NAC on toxic biomaterial is thought to occur through multiple pathways. In the present study, NAC alleviated intracellular ROS elevation on/around β-TCP. Several hypothetical pathways underlying NAC-mediated detoxification
has been proposed in previous studies. The first involves the direct *in situ* scavenging of free radicals. NAC can directly scavenge extracellular ROS [30] and toxic compounds [29] originating from the material with its functional base, a sulfhydryl group. Electron spin resonance spectroscopy analysis revealed the rapid reduction of free radical levels within NAC-supplemented PMMA resin [10]. The second pathway involves that NAC releases from the material and reinforces the antioxidant capacity of cells. NAC is easily taken into cells and maintain the cellular redox system by acting as a glutathione supply source [59]. Our previous studies demonstrated that NAC enhanced intracellular glutathione level on the PMMA resin beyond the level on nontoxic substrate, polystyrene, in contrast with the depletion of the cellular glutathione observed on the untreated resin material [10, 60]. Moreover, NAC might have a potential to modulate cellular signaling pathways involved in cell viability and function. It has been suggested that NAC influences redox-sensitive transcriptional factors such as nuclear factor kappa β and activator protein-1, which play a central role in cell survival and differentiation-related signaling pathways [61]. Determination of causal agents of the β-TCP and the NAC-mediated detoxification mechanisms are key questions for future research.

Many tissue forming and immune cells such as osteoblast, periosteal cell, endothelial cell, fibroblast and immunocyte, and the multiple interaction among such cells are involved in bone formation on/around material in local tissue. Also, a local circulatory of blood and tissue fluid works to transport toxic products away during the initial phases of wound healing. The present study showed negative responses of osteoblasts to β-TCP granules and its alleviation by NAC only in culture model. Further investigation of the effects of NAC in animal model and cellular interaction networks will be of great interest for future research. However, we could anticipate that an improvement in the osteoblastic compatibility of β-TCP results in the enhancement of surrounding bone regeneration in term of quantity and quality through acceleration of bone formation and increase in bone volume, hardness and stiffness and strength of bone-material integration. In fact, NAC-mediated detoxification of PMMA-based bone cement resulted in a marked increase in bone-contact area,
volume of surrounding bone formation and biomechanical strength at the bone-material interface, it is called, creation of osseointegration capacity for resin-based bone cement [10].

The biological property of β-TCP granules was improved to the biological significant degree by soaking the material with NAC solution. We would need the further exploration for the most effective method to apply NAC for β-TCP material on the basis of the NAC-mediated detoxification mechanism and the physicochemical property of β-TCP. The results of the present study suggest that application of antioxidant amino acid derivative NAC may offer a new avenue for the biofunctionalization of β-TCP-based materials for bone regeneration. Also, this may provide important information for future breakthroughs in the development of synthetic bone substitutes.

**Conclusion**

Culturing rat calvarial osteoblasts on β-TCP bone substitute granules resulted in suppressions of viability and ALP activity with markedly increase of intracellular ROS level. Induction of apoptosis and intracellular ROS elevation were also observed in osteoblasts cultured on polystyrene underlying β-TCP granules without physical contact. Within the limitation of the model used in this study, pretreatment of β-TCP granules with NAC significantly improved the osteoblastic responses to the material.

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**References**

J Biomater Appl.

16 Lenz, R., Mittelmeier, W., Hansmann, D., Brem, R., Diehl, P., Fritsche, A. and Bader, R. (2009) Response of human osteoblasts exposed to wear particles generated at the interface of total hip stems and
bone cement. J Biomater Appl. **89**, 370-378


Nicotera, P., McConkey, D., Svensson, S. A., Bellomo, G. and Orrenius, S. (1988) Correlation between cytosolic Ca2+ concentration and cytotoxicity in hepatocytes exposed to oxidative stress. Toxicology. 52, 55-63


Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.
Legend

Fig. 1. Cell viability and apoptosis analysis using Annexin V-based flow cytometry in osteoblasts cultured on polystyrene and on beta-tricalcium phosphate (β-TCP) granules with or without NAC pretreatment at 24 hr after seeding. Flow cytometric images are shown at top, and percentages of viable cells (quadrant 3 in upper images), necrotic cells (quadrant 1) are shown at bottom. Poly, polystyrene culture; BT, culture on untreated β-TCP; NBT, culture on β-TCP pretreated with NAC. Data represent mean ± SD (N = 3). **p < 0.01, (Bonferroni).

Fig. 2. Alkaline phosphatase (ALP) staining of osteoblastic culture on polystyrene and on beta-tricalcium phosphate (β-TCP) granules with or without NAC pretreatment at day 5. 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. Poly, polystyrene culture; BT, culture on untreated β-TCP; NBT, culture on β-TCP pretreated with NAC. Data represent mean ± SD (N = 3). **p < 0.01, (Bonferroni).

Fig. 3. (A) Number of attached cells measured by hematocytometer and (B) intracellular ROS level evaluated by DCF-DA fluorophotometry in osteoblasts cultured on polystyrene and on beta-tricalcium phosphate (β-TCP) granules with or without NAC pretreatment at 24 hr after seeding. Poly, polystyrene culture; BT, culture on untreated β-TCP; NBT, culture on β-TCP pretreated with NAC. Data represent mean ± SD (N = 3). **p < 0.01, (Bonferroni).

Fig. 4. A) Cell viability and apoptosis analysis using Annexin V-based flow cytometry in osteoblasts cultured on polystyrene with or without co-incubation in non-contact with beta-tricalcium phosphate (β-TCP) granules with or without NAC pretreatment at 24 hr after seeding. Flow cytometric images are shown at top, and percentages of viable cells (quadrant 3 in upper images), early apoptotic cells
(quadrant 4), late apoptotic cells (quadrant 2) and necrotic cells (quadrant 1) are shown at bottom. B) Intracellular ROS level evaluated by DCF-DA fluorophotometry in osteoblasts cultured on polystyrene with or without co-incubation in noncontact with β-TCP granules with or without NAC pretreatment at 24 hr after seeding. Poly, polystyrene culture; BT, culture on untreated β-TCP; NBT, culture on β-TCP pretreated with NAC. Data represent mean ± SD (N = 3). **p < 0.01, (Bonferroni).