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The inhibition of infection by wound pathogens on scaffold in tissue forming process using N-acetyl cysteine

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Short title: Anti-infection capability of NAC for tissue regeneration

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Prevention of local infection from wound pathogens such as Staphylococci and Streptococci is crucial for tissue regeneration. N-acetyl cysteine (NAC), an antioxidant amino acid derivative, has anti-microbial potential against various species. This *in vitro* study evaluated whether NAC prevented bacterial infection of gingival fibroblasts and osteoblasts on a scaffold. N-acetyl cysteine delayed growth of *Staphylococcus aureus* and *Streptococcus pyogenes* cultured in brain heart infusion (BHI) broth for 12 hr in an almost dose-dependent manner (2.5, 5.0 or 10.0 mM). The number of rat gingival fibroblasts on collagen scaffolds with bacterial co-incubation was less than 30% of that in cultures without bacterial co-incubation at day 7. However, pre-addition of NAC to the scaffold yielded a number comparable with that in culture without bacteria. Fibroblasts on the scaffold with bacterial co-incubation were small, rounded and filled with bacteria and reactive oxygen species. Pre-addition of NAC, however, resulted in fibroblasts similar to those observed in culture without bacterial co-incubation. N-acetyl cysteine completely prevented devastating suppression of alkaline-phosphatase activity and extracellular matrix mineralization in osteoblastic culture on scaffolds with bacterial co-incubation. These results indicate that NAC can functionalize a scaffold with anti-infective capabilities, thus assisting healing of soft and hard tissues.

*Key words:* antioxidant; bone regeneration; Streptococci; multi-functionalization; Staphylococci; tissue engineering
Wound infection is one of the most serious issues in tissue regeneration and biomaterial implantation. Generally, certain types of Staphylococci and Streptococci such as *Staphylococcus aureus* and *Streptococcus pyogenes* are regarded as representative pathogens of wound infection [1, 2]. These bacteria are Gram-positive and accrete as indigenous bacteria on body surfaces, including the skin, nose, genitals, anus and oral cavity. An acute wound arising from a cut, laceration or surgical procedure, provides an opportunity for such bacteria to enter and colonize the underlying tissue, including connective, muscle and bone tissue. Even after rigorous disinfection, the remaining bacteria can continue to grow and infiltrate the underlying tissue through gaps between the flaps of the wound until healing is complete [3, 4]. Implantable medical devices and materials such as artificial skin, pacemakers, heart valves, orthopedics, dental bone-anchored prostheses and bone graft materials are also subject to the bacterial invasion scenario [3-6]. Bacterial contamination of an implantable scaffold results in tissue breakdown and degradation around the implant material.

Oxidative stress, which results from increased levels of reactive oxygen species (ROS) and a distortion of cellular redox balance, is involved in several pathological conditions. Intracellular ROS can be excessively generated due to various exogenous stimuli such as ultraviolet (UV) light, ionizing radiation, physical stimuli, various chemicals and toxins and bacterial infection [7-9], which markedly consumes the cellular antioxidant glutathione (GSH). Due to their high reactivity, ROS can oxidize cellular components such as lipids, proteins, and DNA and thus damage cell structure and integrity [7, 10-13]. In addition, ROS induce apoptosis via a wide variety of cellular pathways, including a release of cytochrome c from mitochondria and lysosome membrane permeabilization [14-17]. Oxidative stress is strongly associated with the phagocytotic process [18-21]. Some bacterial species including *S. aureus* and *S. pyogenes* utilize the phagocytotic process for invasion into cell [22-24]. Recent culture studies demonstrated that phagocytosis-related ROS generation caused intracellular oxidative damage leading to cell death or dysfunction in macrophages, fibroblasts and
N-acetyl cysteine (NAC), an antioxidant amino acid derivative, is used in expectorant in respiratory medicine. This small-molecule compound (molecular weight: 163.19) can directly scavenge free radicals and toxic compounds with its functional moiety, the sulfhydryl group [7, 29]. In addition, NAC is membrane-permeably incorporated into cells and rapidly metabolized into L-cysteine, a precursor of GSH, resulting in maintenance of intracellular redox balance. This strong antioxidant activity of NAC protects cells from oxidative stress caused by various external stimuli [7, 30]. In earlier studies, we demonstrated that NAC detoxified oxidative stress-inducing biomaterials such as polymethyl methacrylate resin (PMMA) and organic and inorganic bone biomaterials, allowing maintenance of cell viability and differentiation in dental pulp cells, fibroblasts and osteoblasts in contact with those materials by reduction of oxidative stress [29, 31-38]. Large quantities of direct bone deposition occurred on NAC-incorporated PMMA bone cement implanted into rat femur bone in contrast with sparse bone formation around PMMA cement alone [33]. These results indicate that the antioxidant properties of NAC can functionalize biomaterial, enhancing its cytocompatibility. Moreover, recent culture studies indicated that NAC also had antimicrobial potential. It was shown that NAC delayed biofilm formation of certain types of fungi and bacteria such as Aspergillus and Fusarium species [39], Escherichia coli [40], Pseudomonas aeruginosa [41], Staphylococcus epidermidis [42], Staphylococcus warneri, Streptococcus pneumoniae, Acinetobacter baumannii and other Gram-positive and negative bacteria on polystyrene and stainless steel surfaces [43]. Other culture studies showed that NAC reduced bacterial and viral adherence on human pharyngeal or oropharyngeal epithelial cells [44, 45].

The unique pharmacological properties of NAC, however, raise the following intriguing questions: 1) does NAC exert an anti-microbial effect on the representative Staphylococci and Streptococci involved in wound infection?; 2) can preparation of a tissue-engineering scaffold with NAC prevent
bacterial infection and related oxidative stress in cells attaching to it?; and 3) if so, can NAC prevent loss of viability and function in both soft and hard tissue-forming cells caused by bacteria and encourage tissue formation on a scaffold, even in the presence of wound-associated pathogens? Clarification of these questions would pave the way for the development of multifunctionalized biomaterials with not only enhanced cytocompatibility, but also anti-infection capabilities. The objectives of this *in vitro* study were 1) to determine whether NAC exerted an anti-microbial effect on *S. aureus* and *S. pyogenes*; and 2) to determine whether addition of NAC to a collagen membrane or spongy scaffold protected fibroblasts or osteoblastic cells cultured on that scaffold from bacterial infection, bacterially induced oxidative stress, cell death and dysfunction under co-incubation with wound pathogens. Furthermore, we sought to further clarify the mechanisms and pathways involved in NAC-mediated anti-infective functionalization.

2. Materials and methods

2.1. NAC preparation

An NAC stock solution was prepared by dissolving NAC powder (Sigma-Aldrich, St. Louis, MO) in HEPES buffer (1 mol/L stock, pH 7.2). The solution for fibroblastic or osteoblastic culture comprised the NAC stock solution mixed with Dulbecco’s Modified Eagle’s medium (D-MEM, Gibco BRL Div. of Invitrogen, Gaithersburg, MD) or alpha-modified Eagle’s medium (α-MEM, Gibco BRL Div. of Invitrogen, Gaithersburg, MD) in a volume ratio of 1 to 9.

2.2. Bacterial strain and culture condition

*Staphylococcus aureus* 209P and *S. pyogenes* GTC 262 were used in the experiment. Each bacterium was grown in 3 ml brain heart infusion broth (BHI, Becton Dickinson, Sparks, MD) in a 12-ml sterile plastic tube capped tightly and incubated at 37°C statically overnight (16–18 hr).

2.3. Effect of NAC on bacterial growth
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Susceptibility in broth culture

Brain heart infusion broth cultures with or without NAC were used for quantitative evaluation of the effect of NAC on bacterial growth. The density of the cell cultures was adjusted photometrically so that they contained approximately $1.0 \times 10^6$ cells/ml prior to their use in the experiment. Untreated bacterial cultures and experimental cultures with addition of 2.5, 5.0 or 10.0 µl NAC (final concentration of 2.5, 5, or 10 mM per 1 ml culture medium) and bacterial culture with addition of 10.0 µl HEPES buffer were prepared for evaluation of bacterial growth in culture over 12 hr incubation. Reagents were added one time at the kickoff point of incubation.

Bacterial growth was quantified by absorbance at 660 nm in the BHI broth using a microplate reader after 4, 8 and 12 hr incubation. Microbial metabolic activity was also quantified by bioluminescence-based adenosine triphosphate (ATP) assay using BacTiter-Glo™ Reagent (Promega Corporation, Madison, WI). After 4, 8 and 12 hr incubation, reagent was added to the bacterial broth culture and luminescent intensity measured using a microplate reader according to the manufacturer’s instructions.

Halo test

The anti-bacterial effect of NAC was also investigated with the Halo test. Filter paper disks 6.0 mm in diameter and impregnated with 30 µl HEPES buffer or NAC solution (final concentration of 2.5, 5, or 10 mM per 30 µl) were placed on BHI agar plates on which 100 µl BHI broth containing approximately $1.0 \times 10^8$ cells/ml had been uniformly smeared in advance. After 24 hr incubation at 37°C, an image analysis was performed using ImageJ (National Institute of Health, Bethesda) to determine growth-inhibitory width, which was defined as the radius of the growth-inhibitory area (mm). Subsequently, subculture streaked with a sample from the growth-inhibitory area was used to determine viability of bacteria in that area.
2.4. Preparation of collagen scaffold

Commercial collagen membrane (Tissue Guide®, KOKEN, Tokyo, Japan) and spongy scaffold (Collaplug®, Zimmer Dental, Carlsbad, CA) made from bovine type I atelocollagen were used as the substrate for fibroblastic and osteoblastic culture. The membrane scaffold was cut into small pieces, uniform in shape and with a surface area of 44.2 mm$^2$ each. The spongy scaffold was trimmed into small pieces uniform in area (44.2 mm$^2$) and thickness (0.3 mm). Each specimen was placed on a culture-grade polystyrene 48-well plate. Immediately before cell seeding, the collagen material substrate was permeated with 50 μl NAC-treatment solution or culture basal media (D-MEM or α-MEM) alone. It was confirmed that the collagen substrate retained sufficient integrity after immersion in culture medium for 21 days to allow subjection to analysis.

2.5. Rat fibroblastic and osteoblastic culture on collagen scaffold with bacterial co-incubation

Fibroblastic cells were obtained from the palatal gingiva of 8-week-old Sprague–Dawley rats. After the animals were sacrificed, the palatal tissue was aseptically removed and washed with 1% phosphate buffered saline (MP Biomedicals, Solon, OH). The tissue was dissected into small pieces and digested with 0.25% collagenase for 12 hr. The liberated cells were collected into D-MEM supplemented with 10% fetal bovine serum and an antibiotic–antimycotic solution 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 4-Na and seeded onto polystyrene, untreated collagen material, or NAC-treated material at a density of $4 \times 10^4$ cells/cm$^2$ in 0.25 mL D-MEM without the antibiotic–antimycotic supplement.

Bone marrow cells isolated from the femurs of 8-week-old male Sprague–Dawley rats were placed in osteoblastic medium consisting of α-MEM supplemented with 15% fetal bovine serum, 50 μg/mL ascorbic acid, $10^{-8}$ M dexamethasone, 10 mM Na-β-glycerophosphate, and 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 $4 \times 10^4$ cells/cm$^2$ in 0.25 mL osteoblastic media without the antibiotic–antimycotic supplement.

After seeding cells on the scaffold, 5 μl bacterial suspension containing $2.5 \times 10^5$ bacteria was added to the fibroblastic or osteoblastic culture where the final concentration of bacteria was $1.0 \times 10^6$ cells/mL. Multiplicity of infection (MOI) in the co-culture was 6.25. The cells and bacteria were cultured for up to 21 days and 70 μl fresh D-MEM or osteoblastic medium without supplemental NAC or antibiotic–antimycotic solution added at 3-day intervals. The study protocols were approved by the Animal Research Committee of Tokyo Dental College (Protocol No. 222604).

2.6. Quantification of viable cell number

To evaluate fibroblastic viability and attachment on the collagen membrane scaffold at days 2 and 7, calcein-AM fluorescent staining (Cell Counting Kit-F, Dojindo Molecular Technologies, Rockville, MD) was used. Calcein-AM is selectively taken into viable animal cells, but not bacterial cells, and hydrolyzed to calcein by intracellular esterase. Immediately before fluorescent staining, cultured collagen specimens were transferred to a new culture plate. After discarding the supernatant from the cultures, the 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 calcein-AM solution 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. Scanning electron microscopy (SEM) of the selected cultures was carried out to confirm the absence of cell remnants on the substrates.

2.7. Cellular ROS Detection
The amount of intracellular ROS production in the fibroblasts at 24 hr after seeding was quantified by fluorometry with 5-(and-6)-carboxy-2’,7’-dichlorofluorescein diacetate (carboxy-DCF-DA) (Invitrogen, Gaithersburg, MD), 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 collecting and staining the cells with carboxy-DCF-DA, 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 values were divided by the number of attached cells in the replicated culture measured with calcein-AM. The selected cultures were subjected to SEM to confirm the absence of cell remnants on the substrates.

2.8. Cellular GSH detection

To quantify total GSH in the culture, DTNB (5,50-dithiobis(2-nitrobenzoic acid)) (Total Glutathione Quantification Kit, Dojindo Molecular Technologies, Inc., Gaithersburg, MD) was used. DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid and GSH disulfide (GSSH). The GSH produced from GSSH by GSH reductase again reacts with DTNB. Therefore, the GSH concentration can be determined by measuring the 2-nitro-5-thiobenzoic acid concentration. Twenty-four hours after seeding, cultured collagen specimens were transferred to a new culture plate. The fibroblasts in the culture were collected and incubated with DTNB and glutathione reductase for 10 min at 37°C. Total cellular GSH concentration was determined by measurement of absorbance at 412 nm. The values were divided by the number of attached cells in the replicated culture measured with calcein-AM. The selected cultures were subjected to SEM to confirm the absence of cell remnants on the substrates.

2.9. Cellular lysosome detection

Lysosome production in the fibroblasts at 24 hr after seeding on collagen scaffold was detected by fluorescent acidotropic probes for labeling and tracking acidic organelles in live cells (Lysotraker™,
Molecular Probes, Inc., Eugene, OR). Immediately before fluorescent staining, cultured collagen specimens were transferred to a new culture plate. After collecting and staining the cells with the probe solution, the fluorescence intensity was measured using a fluorescence plate reader at 422 nm (excitation at 373 nm) against D-PBS as a blank. The values were divided by the number of attached cells in the replicated culture measured with calcein-AM. The selected cultures were subjected to SEM to confirm the absence of cell remnants on the substrates.

2.10. Cell morphology, morphometry and intracellular ROS and lysosome visualization

After 24 hr of culture, the fibroblasts on the scaffold were stained with carboxy-DCF-DA for intracellular ROS or fluorescent acidotropic probes for lysosome. The cells were then fixed in 10% formalin and stained with fluorescent dyes: rhodamine phalloidin (red for actin filament; Molecular Probes, Eugene, OR) for cytoskeleton staining and DAPI (blue for nuclei; Vector, CA) for nuclear staining. Confocal laser scanning microscopy was used to examine cell morphology and cytoskeletal arrangement and to visualize ROS or lysosome production in the cells. A quantitative assessment of cell morphometry (cell area, perimeter and Feret’s diameter) was performed using an image analyzer (ImageJ, National Institutes of Health, Bethesda).

2.11. Quantification of bacterial invasion of cells

Bacterial invasion of fibroblasts on the scaffold was evaluated by the standard antibiotic protection assay previously described [46]. Briefly, after 24 hr incubation, unattached bacteria were removed by washing the culture with D-PBS 3 times. External adherent bacteria were then killed by incubating the culture with D-MEM containing 200 μg/mL metronizazole and 300 μg/mL gentamicin for 1 hr. Subsequently, the cultures were incubated in 0.25 mL sterile distilled water (ddH2O) per well for 30 min and pipetted repeatedly in order to destroy cell membrane and make cellular lysates containing infecting bacteria. Viable bacteria in the lysates were stained with 5-Cyano-2,3-ditolyl-2H-tetrazolium chloride (CTC, Dojindo Molecular Technologies, Inc.,
and their quantity measured using a fluorescence plate reader at 630 nm (excitation at 450 nm) against ddH₂O as a blank.

2.12. Visualization of bacterial invasion of cells
For this analysis only, the bacteria were pre-labeled with CTC before co-incubation with fibroblasts on the scaffold in order to prevent undesirable CTC accumulation in viable cells due to CTC’s non-specific stainability for viable cells. After washing the culture and killing the external adherent bacteria in the culture at 24 hr after seeding as described above, the culture was incubated in calcein-AM solution as a selective dye for living animal cells. Subsequently, the stained culture on the scaffold was fixed in 10% formalin and mounted on a glass plate with an encapsulating material with DAPI for nuclear staining (VECTASHIELD Mounting Medium, Vector, Burlingame, CA). The specimens were observed under confocal laser scanning microscopy.

2.13. Alkaline phosphatase staining
On day 10, osteoblastic cultures on the collagen scaffolds with or without bacterial co-incubation 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 the area of positive staining for alkaline phosphatase (ALP), defined as: [(stained area/total substrate area) × 100] (%) using ImageJ.

2.14. Mineralizing capability
The mineralizing capability of the osteoblastic cells on the scaffold with or without bacterial co-incubation at days 14 and 21 was determined with the von Kossa stain. The cells were fixed using 50% ethanol/18% formaldehyde solution for 30 min. The cultures were then incubated with 5% silver nitrate under UV light for 30 min. Finally, the cultures were washed twice with ddH₂O and incubated with 5% sodium thiosulfate solution for 2–5 min. The mineralized nodule area, defined as
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[(stained area/total dish area)×100] (%), was measured using ImageJ.

2.15. Statistical analysis

The number of samples was 3 for all the cell culture studies, except for cell morphometry, in which 6 samples were used of the evaluation. An ANOVA was used to assess differences among multiple experimental groups, and, when appropriate, Bonferroni multiple comparison testing was used. The t-test was used to examine differences between two groups; p<0.05 was considered statistically significant.

3. Result

3.1. Effect of NAC on staphylococcal growth

Clouding of the BHI broth containing S. aureus 209P was observed with increase in culture time, while intensive white turbidity was noted at 8 and 12 hr incubation in untreated bacterial culture compared with in media without bacteria (Fig.1A, top images). However, the increase in white turbidity was clearly suppressed by addition of NAC at the starting point of culture. White turbidity after 12 hr culture weakened in an NAC-dose dependent manner, with addition of 5 or 10 mM NAC, in particular, yielding a clear culture at 12 hr incubation. Measurement of white turbidity revealed remarkable growth of bacteria at 4 hr culture, with white turbidity in the culture 150 and 190 times greater at 8 and 12 hr incubation than at the starting point of culture (Fig.1A, bottom line graph). Meanwhile, NAC-added cultures showed an NAC-dose dependent reduction in white turbidity, which was down to one-tenth and one-fifth of that in the untreated cultures at 8 and 12 hr incubation, respectively. Addition of HEPES buffer did not inhibit bacterial growth. Adenosine triphosphate activity in the untreated bacterial culture showed a marked increase at 4 hr incubation, increasing 30-fold at 12 hr incubation compared to that at the starting point of incubation (Fig.1B). However, NAC repressed this increase in bacterial ATP activity with time in an NAC-dose dependent manner.
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The NAC-added cultures showed significantly lower ATP activity than untreated culture at 4, 8 or 12 hr incubation time (Fig. 1B) (p < 0.05, Bonferroni). The level of ATP activity, in particular, remained at half or less of that in the untreated bacterial culture in the 5.0- and 10.0-mM NAC cultures, even after 12 hr incubation.

A growth inhibitory area for *S. aureus* was observed around the edge of the diffusion disks containing NAC in the day-1 BHI agar culture plates, whereas no such area was observed around disks containing HEPES buffer (Fig. 1C, top images). Halo width around disks containing NAC solution increased in an NAC-concentration dependent manner with up to 5.0 mM NAC (Fig. 1C, bottom histogram). The 5.0- and 10-mM NAC disks yielded an approximately 1.0-mm halo width, which was twice that around the 2.5-mM NAC disk (p < 0.05). No halo was detected around the HEPES buffer disks, however. Brain heart infusion agar subculture originating from the growth inhibitory area around NAC-containing disks allowed intensive bacterial growth and colonization with 3 days incubation, regardless of NAC concentration in the disk on the original culture (Fig. 1C, middle images).

3.2. Effect of NAC on fibroblastic viability suppressed by bacteria

The number of attached viable fibroblasts on the scaffold on day 2 of culture was 70% less with *S. aureus* co-incubation than that without bacterial co-incubation (Fig 2) (p < 0.05, Bonferroni). At day 7 of culture, the number of attached fibroblasts on the scaffold remained 75% less with bacterial co-incubation than that with culture without bacteria (p < 0.05), in which the cells showed a 2-fold increase in comparison with that in 2-day culture. However, pre-addition of NAC to the scaffold increased the number of attached viable cells on the scaffold, even with bacterial co-incubation. With pre-addition of NAC, the number of attached viable cells on the scaffold increased by 200% and 370% at days 2 and 7 (p < 0.05), respectively, even with bacterial co-incubation, reaching a level equivalent to that on the scaffold without bacterial co-incubation at day 7 (p > 0.05).
3.3. Effect of NAC on fibroblastic dysfunction and ROS generation induced by bacteria

Fewer attached fibroblasts were observed on the untreated scaffold with *S. aureus* co-incubation than on the scaffold without bacterial co-incubation (Fig. 3A, top images). The fibroblasts cultured on the scaffold without bacterial co-incubation expanded fully, developed cytoskeletal filaments and cellular processes, and exhibited little intracellular ROS, whereas the cells cultured on the untreated scaffold with bacterial co-incubation were round, small, and filled with dense ROS (Fig. 3A, from second top to bottom images). Pre-addition of NAC visibly increased the amount of fibroblastic attachment on the scaffold, even with bacterial co-incubation (Fig. 3A, top images). Relatively wide, large, and stretched cells with weak and sparse ROS were found on the NAC-added scaffold with bacterial co-incubation (Fig. 3A, top images). The area, perimeter, and Feret’s diameter of the cells on the scaffold with bacterial co-incubation were less than one fifth of that on the scaffold without bacterial co-incubation (Fig. 3B). Scaffolds with pre-added NAC exhibited 2.5–3.5 times greater values for these parameters than the untreated scaffold under bacterial co-incubation.

3.4. Effect of NAC on oxidative stress induced by bacteria

Intracellular ROS production in viable fibroblasts in day-1 cultures was 1.6-times greater on untreated collagen scaffold with *S. aureus* co-incubation than on scaffold without bacterial co-incubation (Fig. 4A) (p < 0.05). Pre-addition of NAC reduced bacteria-induced ROS production in cells attached to the scaffold (p < 0.05) down to levels comparable with that on scaffold without bacterial co-incubation (p > 0.05). Fibroblasts cultured on collagen scaffold with bacterial co-incubation for 1 day showed a GSH concentration that was 30% less than that in fibroblasts cultured on scaffold without bacterial co-incubation (p < 0.05) (Fig. 4B), indicating the consumption of cellular GSH by ROS. Fibroblasts cultured on pre-added NAC scaffold with bacterial co-incubation showed a 30% increase in GSH concentration as compared those on untreated scaffold with bacterial co-incubation (p < 0.05), which was comparable to that on scaffold without bacterial
3.5. Effect of NAC on Staphylococcal invasion into cell

Confocal laser microscopic 2-dimensional observation of fibroblastic culture revealed small, rounded, calcein-positive viable fibroblasts on untreated scaffold which were filled with CTC-labeled S. aureus (Fig. 5A). In contrast, little CTC accumulation was seen in fibroblasts on scaffold with pre-added NAC. Three-dimensionally constructed images of individual cells positive for CTC revealed that CTC accumulation was concentrated intensively both intra-and extracellularly on untreated scaffold, whereas few CTC-positive dots were observed on the cellular surface of fibroblasts on NAC-pre-added scaffold (Fig. 5B). The amount of bacteria in the fibroblasts was over 15 times greater on untreated scaffold than on NAC-pre-added scaffold (Fig. 5C) (p < 0.01, t-test).

3.6. Effect of NAC on cellular lysosomal production triggered by bacteria

Lysosomal generation in fibroblasts on the scaffolds at day 1 increased by 4.6 times with S. aureus co-incubation as compared with that in cells on scaffold without bacterial co-incubation (Fig.5D) (p < 0.05). Bacterial-reactive generation of lysosome in fibroblasts on the scaffold was markedly reduced by pre-addition of NAC (p < 0.05), which remained 1.8 times greater than that in cells on scaffold without bacterial co-incubation (p < 0.05).

3.7. Effect of NAC on osteoblastic differentiation suppressed by bacteria

In contrast to an extensive ALP-stained osteoblastic culture on the collagen spongy scaffold without S. aureus co-incubation, very little ALP activity was detected on untreated scaffold with bacterial co-incubation at day 10 (Fig. 6A, top images). Pretreatment of scaffold with NAC, however, resulted in a marked restoration of ALP activity. The percentage of the ALP-positive area was greater than 45% on scaffold without bacterial co-incubation, but less than 3% on untreated scaffold with bacterial co-incubation (Fig. 6A, histogram) (p < 0.05). However, pre-addition of NAC to the
Biomaterials scaffold yielded an up to 50% ALP-positive area, which compared favorably with that on scaffold without bacterial co-incubation (p > 0.05).

Detection of von Kossa-positive mineralized matrix was sparse on untreated scaffold with bacterial co-incubation at day 14 (Fig. 6B, top images), whereas an extensive mineralized matrix was observed on scaffold without bacterial co-incubation. The percentage of the von Kossa-positive area at day 14 was under 5% on untreated scaffold with bacterial co-incubation, whereas it was over 50% on scaffold without bacterial co-incubation. (Fig. 6B, histogram) (p<0.05). In contrast, NAC-pre-added collagen scaffold showed an intensive and wide-ranging von Kossa-positive area at day 14, even with bacterial co-incubation (Fig. 6B, top images). Even under bacterial co-incubation, over 55% of the scaffold area became von Kossa-positive with pre-addition of NAC (Fig. 6B, histogram), which was 12-times greater than that on untreated scaffold with bacterial co-incubation (p < 0.05), a level equivalent to that on scaffold without bacterial co-incubation (p > 0.05). Matrix mineralization in the osteoblastic culture on the scaffold remained poor with bacterial co-incubation, even at day 21, in contrast with intensive, dense and extensive von Kossa-positive nodules on the scaffold without bacterial co-incubation (Fig. 6B, top images). The percentage of von Kossa-positive area on the scaffold with bacterial co-incubation at day 21 was higher than that at day 14, but the value still remained 25% or less (Fig. 6B, histogram). In contrast, at day 21 with pre-added NAC scaffold, osteoblastic culture co-incubated with bacteria was intensively stained with von Kossa, showing a more than 90% von Kossa-positive area (p < 0.05), which was comparable with that on scaffold without bacterial co-incubation (p > 0.05).

3.8. Effect of NAC on *S. pyogenes* infection

White turbidity in the *S. pyogenes* cultures at 4, 8 and 12 hr incubation showed a significant reduction with NAC-addition at the starting point of incubation (Fig. 7A) (p < 0.05). Addition of 5.0 or 10 mM NAC consistently reduced white turbidity in the *S. pyogenes* culture to less than 20% of
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that in the untreated culture at 8 and 12 hr of incubation. The number of attached fibroblasts on the scaffold with \textit{S. pyogenes} co-incubation remained at 40% of that in culture without bacteria co-incubation (Fig. 7B) (p < 0.05). However, the number of attached fibroblasts on the NAC-added scaffold was comparable with that in culture without bacteria co-incubation (p > 0.05). The amount of bacteria among the fibroblasts was 75% lower on NAC-added scaffold than on untreated scaffold (Fig. 7C) (p < 0.05).

4. Discussion

The fate of an implantable biomaterial in relation to bacterial infection is generally pictured as a race between microbial growth versus tissue cellular growth and extracellular matrix production on the biomaterial surface [47, 48]. If the race is won by bacteria, the material surface will become rapidly covered by a bacterial biofilm, resulting in hampering of tissue cellular functions. An \textit{in vivo} model might result in expulsion of the bacteria experimentally-added to local tissue due to the action of the host immune system [48]. There have been many attempts to evaluate the anti-microbial capabilities of biomaterials using an \textit{in vitro} culture model. Hitherto, the evaluation of the anti-microbial functionalization of biomaterials has involved the evaluation of its success in either resisting bacterial adhesion and growth [49] or supporting tissue cell adhesion and proliferation without bacterial co-incubation [50, 51]. An earlier study investigated the race for the surface of a biomaterial between bacteria and tissue cells using an \textit{in vitro} co-culture methodology [47]. This culture study using \textit{S. epidermidis} and U2OS osteosarcoma cells demonstrated that tissue cell viability and adherence on a scaffold were reduced with increase in density of co-incubated bacteria and that bacteria inhibited survival, growth and differentiation of tissue-forming cells more severely under static conditions than under culture medium flow conditions simulating local tissue circulation [47]. The present study employed an \textit{in vitro} co-culture method characterized by static co-incubation of bacteria with fibroblastic or osteoblastic cells on a common collagen scaffold as a biomaterial infection model. The MOI of the co-culture model in the present study was 6.25, which was more
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than the MOI (0.005 - 5) in the previous study, in which co-cultured U2OS osteosarcoma cells showed an approximate 90% reduction in the attached cell number on the substrate in comparison with in culture without bacterial co-incubation [47]. This indicates that the co-culture model used in the present study was capable of providing harsh enough conditions for survival and growth of tissue cells.

The pathogenesis of wound infections by indigenous bacteria is largely dependent on bacterial growth activity and toxin production [23, 24], and these pathogenic processes are closely coordinated. Bacterial extracellular substances such as exotoxins and invasins are mainly secreted during the exponential growth phase, facilitating adhesion on and invasion into animal cells [52-54]. Both *S. aureus* and *S. pyogenes*, although more commonly known as extracellular pathogens, have an intracellular niche that protects them from host and antibiotic-based clearance [22-24]. After a cell comes into contact with a bacterium, it can envelop it through the process of binding, invagination and fusion of the plasma membrane, followed by budding off to form an intracellular vesicle, the phagosome [55-57]. The bacteria sometimes try to escape the phagosome after it is engulfed in the cell before it can undergo lysosomal attack in the phagolysosome by producing various toxins or phenotypic switching [58, 59]. Surviving bacteria subsequently replicate themselves in the host cytoplasm while avoiding the cellular digestion system, eventually causing host cell death.

To our knowledge, this is the first study to demonstrate the anti-infective capabilities of NAC in protecting tissue-forming cells such as fibroblasts and osteoblasts from representative wound pathogen infection. Representative staphylococci (*S. aureus*) and Group A beta-hemolytic streptococci (*S. pyogenes*) suppressed gingival fibroblastic viability, attachment and adhesion on a collagen scaffold. The number of viable gingival fibroblasts attached to collagen scaffold co-incubated with bacteria remained at less than 30% of that in culture without bacterial co-incubation at 7 days co-incubation. Fibroblasts attached to the scaffold suffered intensive bacterial
invasion and lysosomal production, and exhibited a rounded and small shape with dense ROS generation and a large amount of GSH consumption, indicating bacterial infection-related oxidative stress. Co-incubation with *S. aureus* exerted a devastating effect on osteoblastic function, including ALP activity and extracellular matrix mineralization, on a collagen spongy scaffold. However, crucially, bacterial infection-induced cell death and dysfunction were almost completely prevented by pre-addition of NAC into the scaffold. Even under co-incubation with *S. aureus*, pre-addition of NAC to the collagen scaffold allowed substantial fibroblastic attachment, adhesion and proliferation to a level equivalent to that in culture without bacterial co-incubation. Addition of NAC allowed attached fibroblasts to escape *S. aureus* invasion and related oxidative stress, and also ameliorated osteoblastic differentiation suppressed by *S. aureus*, where ALP activity and mineralized matrix formation occurred as intensively as in culture without bacterial co-incubation. This indicates the anti-infective capabilities of NAC, even against streptococci.

We hypothesize several pathways for the anti-infective functionalization of scaffold with NAC (as illustrated in Fig. 8). The first pathway involves NAC’s bacteriostatic effects on these pathogens intra- and extracellularly (Pathway 1 in Fig. 8). Bacterial single culture in BHI broth revealed that NAC could reduce the growth of staphylococcus and streptococcus pathogens for 12 hr in an almost dose-dependent manner (2.5, 5.0 or 10.0 mM). However, bacterial growth in pre-added NAC culture proceeded with incubation time, slowly yet steadily. In addition, secondary smear culture streaked from the growth inhibitory area around NAC-containing disks yielded substantial formation of bacterial colonies. These results demonstrate that NAC has anti-microbial properties against these representative staphylococcus and streptococcus pathogens and that these properties are bacteriostatic, not bactericidal, in effect. These results agree well with those of previous studies in which NAC exerted a bacteriostatic effect on other species [41-43]. It is possible that NAC released from a collagen scaffold restricts bacterial proliferation around tissue cells and the scaffold, resulting in a reduction in opportunity for bacterial attachment and invasion. Moreover, NAC incorporated
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into a cell may exert a bacteriostatic effect by helping retrap intracellular bacteria escaping from phagosomes and the cellular digestion system. The precise mechanism of the bacteriostatic effect of NAC, however, remains to be fully clarified. Previous reports indicated that while the inhibitory effect of NAC on bacterial growth covered a wide spectrum, including both Gram-positive and -negative species and multispecies communities, it was not sensitive to specific species [41, 43]. It has been speculated that NAC exerts its anti-microbial potential by competitively inhibiting amino acid (cysteine) utilization in a bacterium or, by virtue of possessing a sulfhydryl group, may react with bacterial cell proteins [41]. Clarification of the mechanism of NAC’s bacteriostatic effect will be great interest for future research.

The other pathways may involve NAC’s anti-oxidant capacity (Pathway 2 and 3 in Fig. 8). It is well documented that prevention of oxidative stress leads to restoration of cellular viability and differentiation in both fibroblasts and osteoblasts [29, 31-36, 38]. Therefore, it is possible that NAC reinforces or compensates the antioxidant capacity of cells on a scaffold by acting as a GSH supply source (Pathway 2 in Fig. 8). As described previously, NAC is membrane-permeably incorporated into cells and rapidly metabolized into L-cysteine, a precursor of GSH. The cells on an NAC-added scaffold may therefore be able to withstand bacteria-induced oxidative stress. In this study, GSH levels in cells on NAC-added scaffold with bacterial co-incubation were comparable to those in cells without bacterial co-incubation. In addition, a marked elevation of intracellular ROS due to *S. aureus* infection was prevented in fibroblasts in NAC-added culture with bacterial co-incubation. Our previous studies revealed that addition of NAC to PMMA resin material resulted in an increase in cellular GSH level and a reduction in cellular ROS generation in fibroblasts or osteoblasts compared with on polystyrene substrate or the original material without added NAC [33]; addition of NAC resulted in restoration of cellular viability and function suppressed by the original material [33, 36]. Scavenging of cellular ROS, both directly by intracellularly incorporated NAC (Pathway 3 in Fig. 8) and indirectly via GSH supplement, may contribute to prevention of cellular collapse and
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dysfunction due to bacteria-induced oxidative stress and support the cellular digestion system.

Numerous toxins are also involved in the establishment and progress of infection by staphylococcal and streptococcal pathogens. For example, *S. aureus* produces many types of colonization factors, including protein A, teichoic acid and fibronectin binding factor, exotoxins, such as enterotoxin, TSST-1, hemolysin, and leucocidin, and enzymes such as coagulase and protease as pathological agents [2]. Pathological streptococci also produce many types of exotoxic and enzymatic causal agents [1]. The influence of NAC on these the bacterial causal agents may involve more than bacteriostasis and an anti-oxidative effect; it might also be involved in reduction of bacterial invasion and alleviation of bacterially induced cellular oxidative stress. It will be of great interest to investigate the effect of AADs on bacterial causal agents in future research.

Moreover, possible cellular signaling pathways in association with alleviation of bacteria-induced oxidative stress by NAC should be discussed. It has been hypothesized that NAC directly modulates intracellular signaling pathways by affecting the cysteine residuals of redox-sensitive transcriptional factors such as activator protein-1 and nuclear factor kappa B (NF-kB) [7, 60], which are involved in the regulation of cellular survival, proliferation and differentiation [7, 61-63]. The role of NAC in permitting cellular survival and differentiation even under bacterial challenge requires further investigation.

Tissue damage may occur no just as a direct result of the pathogenicity of bacteria, but also due to inflammatory response via the immunological system. In response to bacterial contamination, immunological cells extracellularly release ROS to decompose the pathogens, which, however, also leads to oxidative damage to the surrounded tissue. Moreover, inflammatory reaction is initiated and progresses as a result of proinflammatory cytokine production from various cells in local tissues and their interactive network. Our previous studies demonstrated that NAC alleviated cellular
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inflammatory responses to certain toxic agents [31, 64]. In addition, oxidative stress on fibroblasts induced by hydrogen peroxide was completely eliminated by application of NAC [32]. Therefore, NAC might also exert anti-inflammation capabilities to alleviate tissue/cell damage by immunological reaction against bacterial contamination. Likewise, bone formation in local tissues on or around a biomaterial results from multiple interactions among various types of cell, including osteoblasts, periosteal cells, endothelial cells, fibroblasts and immunocytes. NAC can detoxify various bone substitutes and ensure cellular viability and function on those materials [31, 33, 65]. Taken together, this suggests that NAC has multiple functions capable of simultaneously overcoming various obstacles that might lead to impairment of bone regeneration on a biomaterial. If so, this would provide a new perspective on the development of multifunctional bone biomaterials.

5. Conclusion

NAC delayed the growth of representative wound pathogens S. aureus and S. pyogenes in a dose-dependent manner both in BHI broth and on agar. Rat gingival derived fibroblasts and bone marrow-derived osteoblasts were cultured on untreated and NAC-added collagen scaffolds under static co-incubation with S. aureus or S. pyogenes. Addition of NAC to the scaffold resulted in protection of cells from bacterial infection, related oxidative stress and bacteria-induced impairment of fibroblastic viability, attachment, adhesion behavior and osteoblastic differentiation. It was postulated that two different pathways underlie the anti-infective capabilities of NAC: (1) extra-and intracellular bacteriostasis, and (2) scavenging of cellular ROS generated in response to bacterial attachment and infection both directly and indirectly via glutathione supplement. These results indicate that NAC can functionalize a scaffold with anti-infective properties via multiple mechanisms, thus enhancing healing of both soft and hard tissues.

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**References**


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Figure 1
Figure 2

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Calcein intensity

 UT CM  UT CM  NAC CM  UT CM  UT CM  NAC CM

with S. aureus on  with S. aureus on
Fibroblastic culture  Fibroblastic culture
Day 2  Day 7

*
Biomaterials

**A**

**Day 1 Fibroblastic culture** with *S. aureus* on

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**B**

![Graphs showing measurements](image)

**Figure 3**
**Figure 4**

Panel A: Relative cellular ROS level with *S. aureus* on fibroblastic culture Day 1.

Panel B: Cellular glutathione level (µM) with *S. aureus* on fibroblastic culture Day 1.
A. Fibroblasts co-cultured with *S. aureus* on UTCM and NACCM.

B. Fibroblasts co-cultured with *S. aureus* on UTCM and NACCM.

C. Relative infection level (intracellular CTC intensity) for UTCM and NACCM.

D. Relative lysosome level for UTCM and NACCM with *S. aureus* on Day 1.

Figure 5.
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Figure 6

A

Osteoblastic culture with S. aureus on

Day 10 ALP

UTCS UTCS NACCS

UT CS UT CS NAC CS

ALP-positive area (%) with S. aureus Day 10

B

Osteoblastic culture with S. aureus on

Day 14 Day 21

UTCS UTCS NACCS UTCS UTCS NAC CS

von Kossa-positive area (%) with S. aureus Day 14 with S. aureus Day 21

Figure 6
Figure 7
Biomaterials

Figure 8
Biomaterials

Legends

Figure 1 (A) Amount of *S. aureus* in BHI broth with or without various concentrations of NAC (2.5, 5.0 or 10.0 mM) or 10 μl HEPES buffer at 4, 8 and 12 hr incubation. Top: images of cultures and media without bacteria; Bottom: sequential line graph of 660 nm absorbance of culture. Data represent mean ± SD (N = 3). (B) Metabolic activity of *S. aureus* in BHI broth with or without various concentrations of NAC (2.5, 5.0 or 10.0 mM) at 4, 8 and 12 hr incubation. Data represent mean ± SD (N = 3). (C) Results of Halo test with paper disks impregnated with 30 μl HEPES buffer or NAC solution (final concentration of 2.5, 5, or 10 mM per 30 μl) on BHI agar culture. Images of disks on culture after 24 hr incubation; images of subculture streaked with sample from growth-inhibitory area around disk and histogram of growth inhibitory width are shown at top, middle and bottom, respectively. Data represent mean ± SD (N = 3). *p < 0.05, significant difference compared with culture on untreated collagen scaffold with bacterial co-incubation (Bonferroni correction).

Figure 2 Attached viable cell number quantified by calcein fluorophotometry in fibroblastic cultures on collagen membrane scaffold with or without NAC pre-addition and with or without *S. aureus* co-incubation at days 2 and 7. Data represent mean ± SD (N = 3). *p < 0.05, significant difference compared with culture on untreated collagen scaffold with bacterial co-incubation (Bonferroni correction).

Figure 3 (A) Representative triple-stained confocal laser microscopic images of fibroblastic cultures on collagen membrane scaffold with or without NAC pre-addition and with or without *S. aureus* co-incubation at day 1 using rhodamine for actin filaments (red), DAPI for nuclei (blue) and 2',7'-dichlorofluorescein diacetate (DCF-DA) for intracellular ROS (green) (top panels). Colonial appearance of culture (top panels, low magnification, bar = 50 μm), cell morphology, intracellular ROS distribution and merger in same cells (top panels, high magnification, bar = 20 μm) are shown. (B) Cell morphometric evaluations were performed by image analysis (histograms at bottom). Data
Biomaterials represent mean ± SD (N = 6). *p < 0.05, significant difference compared with culture on untreated collagen scaffold with bacterial co-incubation (Bonferroni correction).

Figure 4 ROS level (A) and cellular glutathione level (B) of fibroblastic cultures on collagen membrane scaffold with or without NAC pre-addition and with or without S. aureus co-incubation at day 1. Data represent mean ± SD (N = 3). *p < 0.05, significant difference compared with culture on untreated collagen scaffold with bacterial co-incubation (Bonferroni correction).

Figure 5 (A) Representative confocal laser microscopic images of calcein-stained fibroblasts (green) on collagen membrane scaffold with or without NAC pre-addition and co-incubated with S. aureus labeled with CTC (red) before seeding at day 1. Colonial appearance of attached viable cells, CTC accumulation in same cells and merger (bar = 20 μm) are shown. (B) Representative 3-dimensional reconstruction images from confocal laser microscopic observation of calcein and DAPI-stained fibroblasts (green for cytoplasm and blue for nucleus) on collagen membrane scaffold with or without NAC pre-addition and co-incubated with S. aureus labeled with CTC (red) before seeding at day 1. (one side of 3-dimensional arrow = 10 μm) (C) Quantification of CTC-positive bacteria in fibroblasts on collagen membrane scaffold with or without NAC pre-addition and co-incubated with S. aureus at day 1. Data represent mean ± SD (N = 3). **p < 0.05, significant difference (Student’s t-test). (D) Intracellular lysosomal level of fibroblastic cultures on collagen membrane scaffold with or without NAC pre-addition and with or without S. aureus co-incubation at day 1. Data represent mean ± SD (N = 3). *p < 0.05, significant difference compared with culture on untreated collagen scaffold with bacterial co-incubation (Bonferroni correction).

Figure 6 Alkaline phosphatase staining on day 7 (A) and von Kossa staining on days 14 and 21 (B) of osteoblastic cultures on collagen sponge scaffold with or without NAC pre-addition and with or without S. aureus co-incubation. Top panels show representative images of ALP-stained or von
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Kossa-stained cultures, respectively. Bottom histogram shows percentage of ALP-positive area or von Kossa-positive area to overall culture area measured using digital image analyzer. Data represent mean ± SD (N = 3). *p < 0.05, significant difference compared with culture on untreated collagen scaffold with bacterial co-incubation (Bonferroni correction).

Figure.7 (A) Amount of *S. pyogenes* in BHI broth with or without various concentrations of NAC (2.5, 5.0 or 10.0 mM) or 10 µl HEPES buffer at 4, 8 and 12 hr incubation. Sequential line graph of 660 nm absorbance of culture is shown at bottom. Data represent mean ± SD (N = 3). (B) Results of attached viable cell number quantified by calcein fluorophotometry in fibroblastic cultures on collagen membrane scaffold with or without NAC pre-addition and with or without *S. pyogenes* co-incubation at day 2. Data represent mean ± SD (N = 3). *p < 0.05, significant difference compared with culture on untreated collagen scaffold with bacterial co-incubation (Bonferroni correction). (C) Quantification of CTC-positive bacterial amount in fibroblasts on collagen membrane scaffold with or without NAC pre-addition co-incubated with *S. pyogenes* at day 1. Data represent mean ± SD (N = 3). **p < 0.05, significant difference (Student’s t-test).

Figure.8 Proposed mechanism underlying anti-infective functionalization of scaffold by addition of NAC. Three different pathways proposed: bacteriostasis (Pathway 1) and anti-oxidative capabilities giving cell protection (Pathways 2 and 3) from bacterial oxidative stress are schematically described. See Discussion in main text for detailed description of these pathways.