Original Article

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Biocompatibility of a chitosan-derived hemostatic agent with human alveolar osteoblasts

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Objective: To evaluate the biocompatibility of a Calcium Alginate/N,O-carboxymethylchitosan (CA/NOCC) hemostatic sponge with primary human alveolar osteoblasts (hAOBs).

Materials and Methods: The primary hAOBs, isolated from alveolar bone chips, were cultured with or without the CA/NOCC sponge. Cell morphology was assessed by scanning electron microscopy, while cell proliferation was determined by MTT assay. The effects of the CA/NOCC sponge extract on osteoblastic phenotypes were evaluated with alkaline phosphatase activity and matrix mineralization assays.

Results: Primary hAOBs, cultured with the CA/NOCC sponge, showed comparable cell morphology to the control cells with minimal cell attachments to the material surface. In addition, the proliferative ability of the cells was relatively unaltered. Moreover, the hAOBs cultured in the osteogenic CA/NOCC extract exhibited a comparable ALP activity level, while the extent of mineral deposition was slightly lower than the control.

Conclusion: Our results indicated that the CA/NOCC hemostatic sponge is biocompatible to hAOBs and could potentially be a promising material for bleeding control in the jaw bone.

Keywords: calcium alginate, cell morphology, cell proliferation, matrix mineralization, N,O-carboxymethylchitosan, osteoblasts

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Introduction

Excessive bleeding is one of the most serious and potentially life-threatening complications in oral surgery. Prompt identification of the cause and proper methods for rapid hemorrhagic control must be undertaken for the safety of the patients. The development and use of various types of hemostatic agents have resulted in significant decreases in the morbidity and mortality rates in emergency medical managements and surgical operations. Until today, the search is still ongoing for a novel hemostatic agent that should be biocompatible, biodegradable, non-immunogenic, widely applicable, affordable and, mostly importantly, efficient for bleeding control in both normal and coagulopathic conditions [1]. In oral surgical procedures, Gelfoam and Surgicel are the most commonly used passive hemostatic materials from their ease of accessibility and usage [1, 2]. However, there have been reports of the potential adverse effects of these two materials and their limited efficiency when used in patients with impaired coagulation cascade [2].

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Chitosan (CS) is a natural polymer which has gained increasing acceptances as an effective hemostatic agent [3] and a biomaterial developed for a variety of uses in dentistry [4]. It is derived from the process of N-deacetylation of chitin, a biopolymer which can be abundantly extracted from the shells of marine animals, i.e. shrimp and crabs. CS has been reported with excellent properties of biocompatibility, biodegradability, antimicrobial ability, wound healing promotion and hemostatic capability [3, 4]. Most importantly, the hemostatic efficacy of CS in coagulopathic conditions has been demonstrated in a number of studies [3, 5, 6]. However, the insolubility of CS in water and neutral pH range may limit the development of CS-based biomaterials for wider medical applications. Thus, several water-soluble CS derivatives were prepared, and they have shown improved material properties and hemostatic capabilities, compared to chitosan [7-9].

N,O-carboxymethylchitosan (NOCC) is one of the water-soluble derivatives of chitosan, prepared by the carboxymethylation reaction, resulting in a biomaterial with a negatively-charged surface ($-COO^-$). Janvikul et.al. [9] have shown that this hydrophilic and biocompatible material enhanced platelets activation/aggregation and *in vitro* blood coagulation, and it also demonstrated a higher hemostatic efficiency than chitosan. It has been used in the development of various CS-based biomaterials to enhance the functionalities in various aspects of clinical applications [7]. Furthermore, when combined with sodium alginate and crosslinked with calcium chloride, the prepared Calcium Alginate/N,O-carboxymethylchitosan (CA/NOCC) hemostatic sponge demonstrated biocompatibility, degradability [10-12] and superior hemostatic efficiency, when compared to SPONGOSTAN® [11, 13].

However, this CA/NOCC biomaterial has never been tested as a potential hemostatic agent for oral tissues. Studies have shown varying cell responses towards biomaterials among primary and permanent cell lines. Despite the limited subculturing cell passages and the heterogeneity of primary cells, they are still believed to be a suitable model to replicate *in vivo* responses [14]. Therefore, in this study, we aimed to evaluate the biocompatibility of the CA/NOCC sponge to primary human alveolar osteoblasts (hAOBs) isolated from the jaw bone. Morphological changes of the cells were determined by scanning electron microscopy, and cell proliferation rate was assessed by MTT assays. In addition, the effects of CA/NOCC on hAOBs functions were evaluated by alkaline phosphatase activity and matrix mineralization assays.

Materials and Methods

Preparation of Calcium Alginate/N,O-carboxymethyl chitosan (CA/NOCC) sponge

N,O-carboxymethylchitosan (NOCC) was synthesized in our lab according to the methods previously described [8]. The Calcium Alginate/N,O-carboxymethylchitosan (CA/NOCC) sponge was developed by the Na^+-Ca^{2+} exchange procedure between sodium alginate (SA; FMC Biopolymer, Philadelphia, USA) and calcium chloride solution ($CaCl_2$; Fluka, Missouri, USA) [11, 12]. Briefly, the 2.5% (wt/vol) highly viscous aqueous solution of 0.5 g NOCC and 2.0 g SA in 100 ml deionized water was poured into molds and lyophilized to produce sponge-like pads (1 mm in thickness). Subsequently, the water-soluble sponges were individually immersed into the gently-stirred 10%wt. aqueous $CaCl_2$ solution for 1 hour to produce the water-insoluble pads. The pads were then removed, washed with deionized water and ultimately freeze-dried to yield the chitosan derivative-based hemostatic material. The CA/NOCC pad was cut into circular pieces (6 and 10 mm in diameters) which were then sterilized by ethylene oxide gas prior to use (Figure 1A).

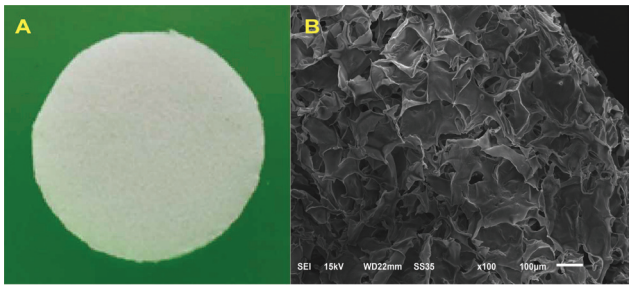

Figure 1 Morphology of the CA/NOCC Sponge. (A) Physical appearance, (B) SEM micrographs of the CA/NOCC sponge. Scale bars in B = 100 µm. CA/NOCC, Calcium Alginate/*N,O*-carboxymethylchitosan.

Human primary osteoblasts isolation and culture

The study protocol received approval from the Ethics Committee of the Faculty of Dentistry, Srinakharinwirot University, Thailand (DENTSWU-EC03/2561).

Primary human alveolar osteoblasts (hAOBs) were isolated from alveolar bone chips obtained during impacted tooth removal. The bone chips were immediately placed in Dulbecco's Modified Eagle Medium/Ham's F-12 (Advanced DMEM/F-12 with high glucose (Life Technologies Corporation, Grand Island, NY, USA), washed with sterile phosphate buffer saline (PBS), and placed in 35 x 10 mm cell culture dishes (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) containing a minimal amount of culture medium, i.e. Advanced DMEM/F-12 supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (Invitrogen), 100 IU/ml of penicillin, 100 ug/ml of streptomycin, and 5 ug/ml of amphotericin B (Invitrogen). The culture dishes were placed in a 37°C humidified incubator with 5% CO_2 atmosphere. The outgrowth of hAOBs from the bone chips were constantly monitored, and the culture medium renewal was done once a week. When approximately 80% of cell confluency was reached, the cells were detached from the dishes using 0.25% Trypsin-EDTA (Invitrogen) and plated into 100 x 21 mm cell culture dishes (Nunc,) for cell population expansion. The cells from passage number 1-5 were used in this study.

CA/NOCC preparations for biocompatibility tests

Direct cell-CA/NOCC contact: The hAOBs were seeded onto the CA/NOCC sponge and cultured to evaluate the effects on the morphology and proliferative capability of the cells.

CA/NOCC culture-medium extract: This method was utilized in the alkaline phosphatase (ALP) activity and the matrix mineralization assays. The CA/NOCC extract was prepared by immersing the CA/NOCC sponge (10 mm in diameter and 1 mm thickness) in the complete culture medium at the ratio of 1 piece of Ca/NOCC sponge to 2 ml of culture medium. The CA/NOCC extract tube was incubated at 37°C with 5% CO_2 atmosphere for 24 hours. For use in hAOBs culture, the extract was aliquoted into a new tube and centrifuged at 12,000 x g for 10 minutes. The supernatant was transferred to a new tube and warmed to 37°C before adding it to the cell culture wells. Fresh culture medium, at the same amount as taken, was added to the CA/NOCC extract tube and further incubated to generate the CA/NOCC extract for future culture medium changes in the assays.

Cell morphology assessment

The effects of CA/NOCC sponge on cell morphology were investigated by scanning electron microscopy. Round cover glasses (13 mm in diameter) were placed into the 24-well cell culture plate (Nunc). The hAOBs were plated onto the cover glasses (control group), or seeded on the CA/NOCC sponge (size 10 mm in diameter), placed on the cover glasses (experimental group), at the density of $6x10^4$ cells/well/ml of culture medium. Following the culture plate incubation in 5% CO_2 atmosphere at 37°C for 72 hours, the cells were rinsed with PBS and fixed with 2.5% glutaraldehyde in deionized distilled water at room temperature for 4 hours. Next, the cells and CA/NOCC sponge on the cover glasses were

rinsed with PBS for 10 minutes, dehydrated in gradient increase of ethanol from 25%-100% (25%-50%-70%-100%, each step for 10 minutes), and fixed with hexamethyldisilazane (HDMS, Sigma-Aldrich, Saint Louis, MO, USA) for 15 minutes in the fume hood. The samples were then left to dry at room temperature before assessment of cells morphology by a scanning electron microscope (JSM-6510LV, JEOL Ltd., Tokyo, Japan).

Cell proliferation assay

Human AOBs were trypsinized and plated at the density of $2x10^4$ cells/well/ml of culture medium in a 24-well cell culture plate (Nunc) (control group), or onto the CA/NOCC sponge (size 6 mm in diameter) placed in the wells (experimental group). The culture plates were then incubated at 37°C with 5% CO_2 for 24 hours to allow cell attachment. Cell viability on days 1, 3 and 6 were evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay. MTT powder (USB, Cleveland, OH, USA) was dissolved in PBS to the final concentration of 5 mg/ml. The MTT/PBS solution was then mixed with serum-free culture medium at the ratio of 1:10 (vol/vol), and 550 µl of the solution mix was added to each well. Following the incubation of the plate at 37 °C with 5% CO_2 for 2 hours, the solution in each well was carefully aspirated, then 500 µl of dimethyl sulfoxide (DMSO; Vivantis Technologies, Selangor Darul Ehsan, Malaysia) was added, and the plate was kept in a rocking motion at room temperature for 30 minutes for complete dissolution of the formazan crystals formed. The solution was transferred to a 96-well plate, in triplicates, at 100 µl/well, and the absorbance was measured at the wavelength of 550 nm using a microplate reader (Asys UVM340; Biochrom, Cambridge, UK).

Alkaline phosphatase (ALP) activity assay

The cells were plated at the density of $2x10^4$ cells/well/ml of culture medium in 24-well cell culture plates (Nunc), which were then incubated at 37°C with 5% CO_2 for 24 hours. Following hAOBs attachment, the culture medium in the wells of the test group was replaced with 1 ml of CA/NOCC extract, while the cells cultured in regular medium were used as the control. Upon cell confluency, the refreshed culture medium and the CA/NOCC extract were supplemented with 50 µg/ml of ascorbic acid, 5 mM of β – glycerophosphate and 10 nM of dexamethasone. At days 4, 8 and 14 following osteogenic induction, the culture medium and CA/NOCC extract were removed from the wells. The cells were rinsed with PBS and incubated on ice for 5 minutes in 200 µl of ice-cold IP Lysis Buffer (Pierce™, Thermo Fisher Scientific, Rockford, IL, USA). The cell lysate was transferred to a microcentrifuge tube and centrifuge at 13,000 x g for 10 minutes at 4°C to pellet the cell debris. The supernatant was transferred to a new microcentrifuge tube for protein concentration and ALP activity analyses.

Total protein concentration in 25 µl of cell lysate was determined by incubating with 200 µl of working reagent from Piece™ BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) in a 96-well plate, at 37°C for 30 minutes. The plate was then cool to room temperature and placed in a microplate reader (Asys UVM340; Biochrom) to measure the absorbance at 562 nm.

To measure the ALP activity levels, 25 µl of cell lysate were added in triplicates along with 50 µl of Alkaline phosphate yellow substrate (P7998, Sigma Aldrich, Saint Louis, MO, USA) in a 96-well plate. The plate was then incubated, protected from light, at room temperature for 15 minutes, and the reactions were stopped by adding 50 µl of 3M NaOH. The levels of the reaction product, p-Nitrophenol, were measured at the absorbance of 405 nm (Asys UVM340; Biochrom), and the concentrations were calculated based on the standard curve prepared from known concentrations of p-Nitrophenol (HiMedia Laboratories, Nashik, India). ALP activity was shown as the p-Nitrophenol concentration normalized to total amount of proteins.

Matrix mineralization assay

The hAOBs were trypsinized, plated at a density of 2 x 10^5 cells/35-mm dish and cultured in 2 ml of Advanced DMEM/F-12 supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml of penicillin, 100 ug/ml of streptomycin, and 5 ug/ml of amphotericin B. The culture dishes were placed in the incubator at 37°C with 5% CO_2 for 24 hours to allow cell attachment, after which, 2 ml of CA/NOCC extract was replaced in the culture dishes assigned to the test group while the cells cultured in regular medium were used as the control. After 7 days of culture, the cells in the control and test groups were maintained, for up to 4 weeks, in the culture medium or CA/NOCC extract containing 50 µg/ml of ascorbic acid, 5 mM of β – glycerophosphate and 10 nM of dexamethasone, respectively. By the end of each week, the cell/matrix layers were washed with PBS, fixed with 100% methanol, and immersed in 1% Alizarin Red S (ARS: Sigma-Aldridge) to stain the mineral deposits. Following the washing process, the dishes were left to dry and stored at -20°C before proceeding to dye extraction.

The amount of mineralization was determined by quantification of the ARS stain using the 10% (vol/vol) acetic acid extraction method [15]. Briefly, 800 µL of 10% (vol/vol) acetic acid was added to the ARS stained dishes, which were incubated at room temperature for 30 minutes. Then, the cell/matrix layers along with the extraction solution were transferred to 1.5-ml microcentrifuge tubes, which were vortexed for 30 seconds, sealed with parafilm, heated to 85°C for 10 minutes, and cooled on ice for 5 minutes. The tubes were then centrifuged at 20,000 x g for 15 minutes, and 500 µl of the supernatant was transferred to a new tube where 200 µl of 10% (vol/vol) ammonium hydroxide was added. Next, the absorbance at 405 nm (Asys UVM340; Biochrom) was measured from the 150 µl triplicate aliquots of the solution mix in a 96-well plate.

Statistical analysis

All experimental data were analyzed using GraphPad Prism version 8.3.1 for Windows, (GraphPad Software, San Diego, California USA, www.graphpad.com), and the results are presented as mean ± standard deviation (SD). Normality of the data was determined by using the Shapiro-Wilk Test. Statistical differences between the groups were assessed by Student's t-test, and the value of $P < 0.05$ was considered significant.

Results

Morphology of the CA/NOCC Sponge and hAOBs

The CA/NOCC sponge used in this study is an off-white, flexible material with porous surfaces (Fig. 1A). From the SEM images, the CA/NOCC sponge demonstrates relatively smooth surfaces and the formation of uniformly-layered and interconnected pores (Figure 1B).

The morphological responses of hAOBs following a 72-hour culture with or without the CA/NOCC sponge were analyzed by SEM. When the cells were seeded onto the CA/NOCC material, most of the cells adhered to the cover glass surrounding the sponge rather than on the surface of the porous CA/NOCC sponge. In both the control and test groups, similar cell density of hAOBs exhibiting flat and fusiform cell morphology were observed (Figure 2A, 2C). From the high magnification images, relatively comparable cell surface features along with formation of lamellipodia for surface attachments were observed in the hAOBs cultured with the CA/NOCC sponge and their control cells (Figure. 2B, 2D).

On the surface of the CA/NOCC sponge, only a trace number of attached hAOBs were found. Morphologically, they were mostly round or spheroid-shaped and anchored to the material surface by the formation of short filopodia. The surface of the hAOBs was homogenously covered with small fibrils (Figure 3A-B).

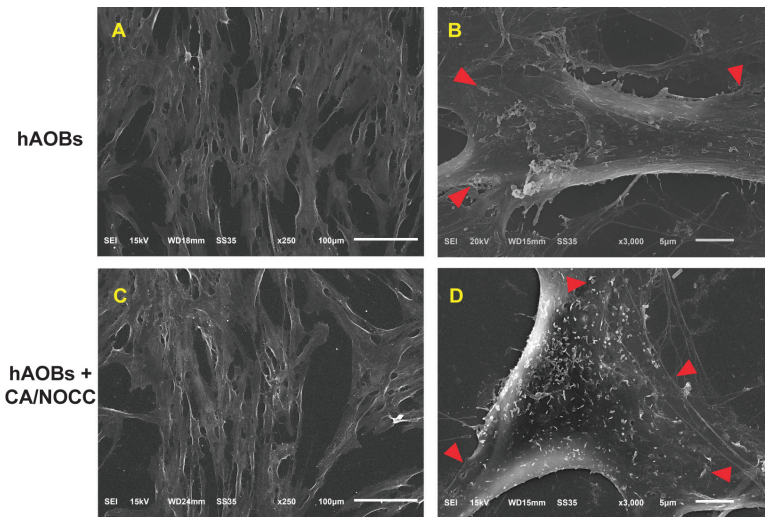

Figure 2 Morphology of primary human alveolar osteoblasts (hAOBs) cultured for 3 days on cover glass (control group) or cultured on cover glass with CA/NOCC sponge (study group) and analyzed by scanning electron microscopy (SEM). (A-B) hAOBs control, (C-D) hAOBs with CA/NOCC. Formations of lamellipodia for cell adhesion were depicted with red arrowheads. Scale bars in A and C = 100 μm; B and D = 5 μm. CA/NOCC, Calcium Alginate/*N,O*-carboxymethylchitosan.

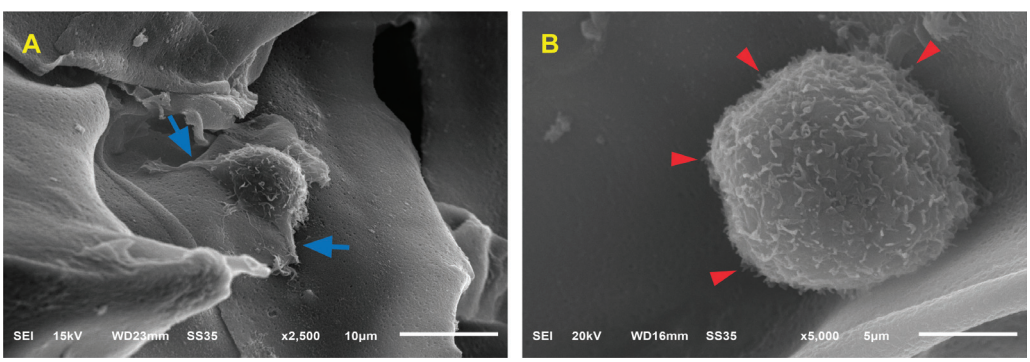

Figure 3 Morphology of primary human alveolar osteoblasts (hAOBs) associated with the surface of CA/NOCC sponge following a 3-day culture and observed by scanning electron microscopy (SEM). (A) Filopodia were shown with blue arrows, (B) Small fibrils were depicted with red arrowheads. Scale bars in A = 10 μm; B = 5 μm. CA/NOCC, Calcium Alginate/*N,O*-carboxymethylchitosan.

Cell Proliferation

The proliferative abilities of the cells cultured with the CA/NOCC sponge were determined by using MTT assay on days 1, 3 and 6. The line graphs (Figure 4A) demonstrated the proliferation patterns of hAOBs from one representative set of experiments. The cell proliferation rates from 5 independent experiments were determined by slope analyses from day 1 to day 6 of culture and were depicted in the bar charts (Figure 4B). We have observed that, when cultured with the CA/NOCC sponge, hAOBs demonstrated comparable proliferative trend and rate (103%) with the control cells (100%).

Alkaline phosphatase activity

Primary hAOBs, cultured in osteogenesis-induced media or CA/NOCC extract at days 4, 8 and 14, were evaluated for the activity levels of ALP. The bar chart (Figure 5A) demonstrated the ALP

activity levels from one representative set of experiments. In both conditions, the activity levels were comparable at days 4 and 8 of culture, while at day 14, ALP activity in hAOBs cultured in the CA/NOCC extract was lower than that of the control. However, when the data from 5 sets of independent experiments were analyzed and demonstrated as percentage of control at each time point, it is apparent that hAOBs cultured in the CA/NOCC extract showed relatively comparable ALP activity levels to those of the control (Figure 5B).

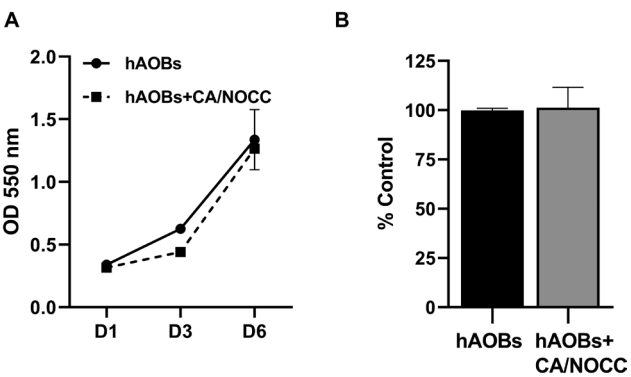

Figure 4 Proliferation of hAOBs was evaluated by MTT assay on days 1, 3 and 6 of cultures with or without the CA/NOCC sponge (control). (A) The line graph plot demonstrates the viability levels, on days 1, 3, and 6 respectively. Data are shown as mean ± standard deviation of absorbance values at 550 nm from one representative experiment. (B) The bar chart demonstrates the proliferation rate from day 1 to day 6 of the cells cultured with CA/NOCC relative to those of the controls. Data are shown as mean ± standard deviation from five independent experiments. CA/NOCC, Calcium Alginate/*N,O*-carboxymethylchitosan; hAOBs, human alveolar osteoblasts.

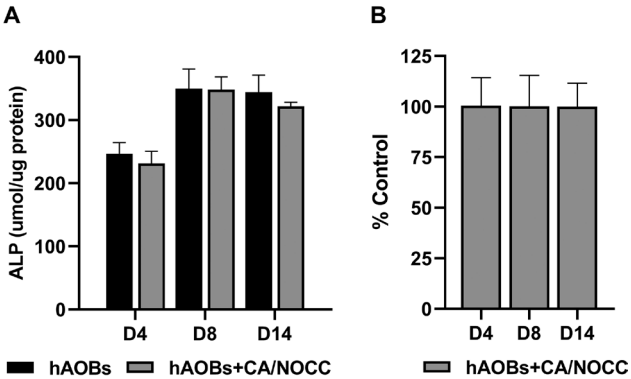

Figure 5 Alkaline phosphatase activity levels in hAOBs cultured in osteogenic-media or -CA/NOCC extract at days 4, 8 and 14. (A) The bar chart shows mean ± standard deviation of the ALP activity from one representative experiment. (B) The bar chart demonstrates the relative ALP activity levels shown as percentage of control (%Control) of hAOBs cultured in CA/NOCC extract to those of the control. Data are shown as mean ± standard deviation from five independent experiments. CA/NOCC, Calcium Alginate/*N,O*-carboxymethylchitosan; hAOBs, human alveolar osteoblasts.

Matrix mineralization

To determine the capability of hAOBs to form mineralized nodules *in vitro*, the cells were maintained in the culture medium or CA/NOCC extract, supplemented with osteogenic inducers, for up to 4 weeks. At the end of each week, *in vitro* mineralization assay was performed by ARS Staining. Mineral depositions in both conditions were initially observed at 2 weeks of culture and gradually increased thereafter (Fig. 6A). Quantitative analyses of ARS stain content at each time point, from 4 independent experiments, were shown as percentages relative to that of the control. At weeks 2, 3 and 4 of culture, hAOBs cultured in osteogenic CA/NOCC extract demonstrated about 5% lower levels of mineralization which were significantly different from the control ($P < 0.05$, $P < 0.01$ and $P < 0.0001$ respectively) (Figure 6B).

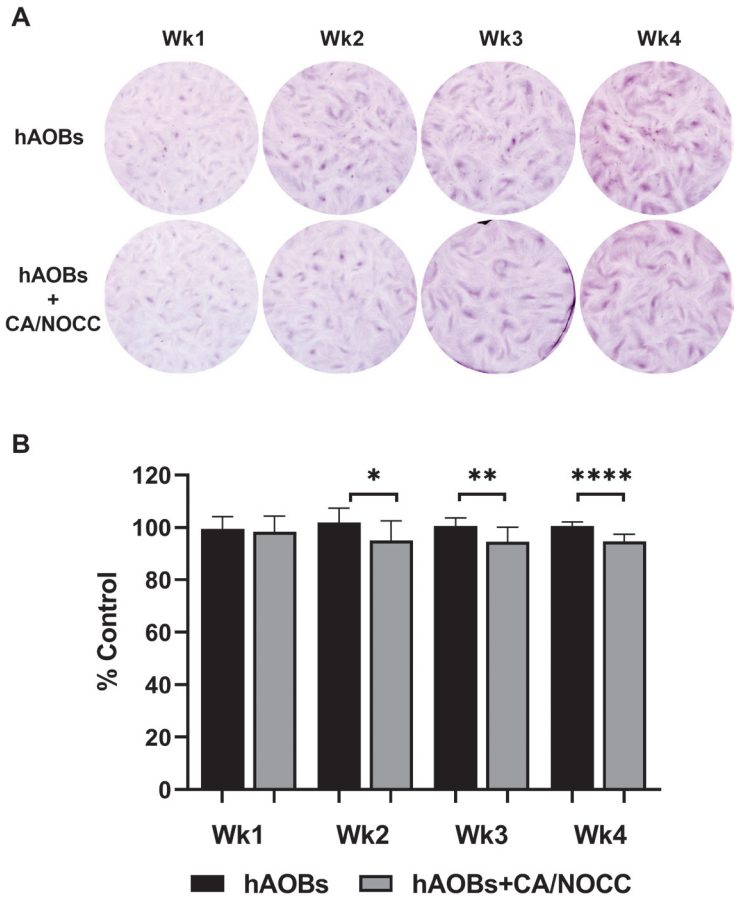

Figure 6 Matrix mineralization assay. Primary hAOBs were cultured in osteogenic-media or -CA/NOCC extract for up to 4 weeks. (A) At the end of each week, the cell/matrices were stained with Alizarin Red S (ARS). (B) The bar chart demonstrates relative levels of the extracted ARS stain, shown as percentage of control (%Control) of hAOBs cultured in CA/NOCC extract to those of the controls. Data are shown as mean ± standard deviation from four independent experiments. *, significantly different at $P < 0.05$; **, significantly different at $P < 0.01$; ****, significantly different at $P < 0.0001$. hAOBs, human alveolar osteoblasts; CA/NOCC, Calcium Alginate/*N,O*-carboxymethylchitosan.

Discussion

To develop a novel biomaterial suitable for clinical uses, its potential harmful effects on cell survival and functions must be initially assessed. Cytotoxicity screening of the material by using established cell lines (e.g. 3T3 or L-929 mouse fibroblasts) provides reliable and reproducible results from their availability, growth efficiency and homogeneity [16]. However, the outcome observed may not fully account for the interspecies differences (i.e. mouse vs human) and most importantly, the potentially different cellular responses compared to those from primary cells isolated from the targeted tissues [16, 17]. In the development process of CA/NOCC hemostatic sponge, we have previously shown that the material was not cytotoxic to L-929 cells [12] and did not cause skin irritations or hypersensitivity reactions in a clinical study [10]. Nevertheless, in order to broaden its usage for bleeding control in the tooth extraction sockets or in other jaw surgical procedures, biocompatibility of the CA/NOCC sponge on hAOBs was evaluated in this study.

When the hAOBs were cultured with the CA/NOCC sponge, the proliferative ability of the

cells was not affected, which is in agreement with our previous report using L-929 fibroblasts [12]. But, most of the hAOBs were attached to the underlying cover glass, while minimal cell adherence was observed on the surface of the CA/NOCC sponge, unlike the high degree of L-929 cells attachment seen in our previous study [12]. The factors affecting the adhering ability of the cells could be, in part, from the intrinsic nature of the biomaterial components i.e. NOCC and CA. It was previously reported that NOCC inhibited the adhesion of substrate-dependent cells e.g. fibroblast, epithelial cells or macrophages [18, 19]. In addition, minimal mouse osteoblasts adhesion was observed on the surface of unmodified alginate hydrogel [20, 21], which has been characterized as an inert material with low protein absorption to support cell attachment [20, 22]. However, it was suggested that the varying degrees of cell adhesion to sodium alginate may resulted from the purity or the relative proportion of guluronic (G)- and mannuronic (M)-block fractions in alginate. Wang et.al. [22] have demonstrated that unmodified alginate with higher content of G-block fractions promoted bone marrow cells adhesion and proliferation, while the M-block fraction may contain anti-adhesive properties. Additionally, we have observed from SEM micrographs that, the spheroid-shaped hAOBs were homogenously covered with small fibrils and attached to the surface of CA/NOCC sponge by forming short filopodia. Our results may be, in part, similar to a study by Li et.al. [23] showing that human osteoblast cell line (MG63) cultured in a chitosan-alginate scaffold crosslinked with $CaCl_2$ demonstrated an enhanced proliferative capacity. Moreover, the cell surface of MG63, adhering to the chitosan-alginate scaffold, was covered with numerous microvilli [23]. Therefore, it could be implicated that, the inconsistencies observed among the studies could potentially be from the cell type-specific responses towards the biomaterial, varying preparations of biomaterials, different cell culture conditions, or methods of assessment.

Regarding the ability of hAOBs to retain osteoblastic phenotypes, when cultured with the CA/NOCC extract, the ALP activity and matrix mineralization capability of the cells were assessed. We have observed that the ALP activities were mainly at their highest levels around day 8 to day 14 of culture. This inconsistency might be from the characteristic variations of hAOBs isolated from different donors. However, those hAOBs cultured in the osteogenic CA/NOCC extract exhibited comparable ALP activity levels, at every time point, when compared to those cultured in the osteogenic media. Moreover, the formation of mineralized nodules in both conditions were initially observed at week 2 of culture, which corresponds to the previous reports showing that mineralization in alveolar bone cell culture occurs after the peak of ALP activity [24, 25]. Interestingly, the extent of mineral deposits by hAOBs cultured in the osteogenic CA/NOCC extract were approximately 5% lower than that of the control, in weeks 2, 3 and 4 of culture. The slightly lower level of mineralization observed in hAOBs exposed to the CA/NOCC extract, may possibly be from the effect of CA/NOCC on the expression levels of other osteoblast markers, mineralization-inducing or -inhibiting proteins [26, 27]. Nevertheless, the *in vitro* mineralization amount difference of 5% in the CA/NOCC group, could be considered very minimal and may not be of clinical significance. Thus, future *in vivo* study of the CA/NOCC sponge is warranted to evaluate its biodegradability and its potential influence on bone or tooth socket healing.

The CA/NOCC hemostatic sponge in this study was prepared from the viscous solution mixture of SA and NOCC, casted and freeze-dried in the mold. The dry sponge was then immersed in $CaCl_2$ solution, where the Na^+-Ca^{2+} exchange process took place, resulting in the formation of CA/NOCC sponge [12]. To compare the outcome

of this study to previous reports, certain limitations were encountered, which include different biomaterial components and preparations, cell types, culture conditions and methods of analyses. Therefore, the previously reported biocompatibility tests of NOCC and CA were reviewed. Studies have shown excellent cytocompatibility of NOCC to various cell types, including osteoblasts [28-30]. Moreover, its reported biocompatible, biodegradable, antimicrobial, anticancer and hemostatic properties along with its ability in wound healing promotion, has deemed it an appealing material for various biomedical applications [7, 11, 12].

Alginate, a natural anionic polysaccharide which can be abundantly extracted from seaweed, has been demonstrated with hemostatic capability when combined with calcium or sodium [31]. Its outstanding properties of biocompatibility, biodegradability, hydrophilicity, wound healing support and inexpensiveness have attracted wide interests in using alginate for tissue engineering and drug delivery purposes [32]. However, there have been reports of the cytotoxic effects of calcium alginate (CA), from the high-level release of calcium ions (Ca^{2+}), that significantly altered the cell morphology and impaired the proliferative abilities of fibroblasts and epidermal cells [33-35]. Previously, we have shown that the degraded product of the CA/NOCC sponge in PBS was sodium alginate, which could possibly be from the release of Ca^{2+} in exchange with sodium ion in PBS [12]. However, by using MTT assays, we have observed minimal cytotoxic effects of the CA/NOCC sponge on hAOBs. Moreover, their osteoblastic phenotypes, determined from the ALP activity levels and matrix mineralization ability, were somewhat maintained. Our results were in agreement with the study by Chen et.al. [36], which demonstrated that the CA scaffold can promote primary human osteoblast ingrowth, differentiation, and mineralized tissue formation. In another study, the viability, proliferation and mineralization capabilities were maintained in SaOS-2 cells, a human osteosarcoma cell line, encapsulated in the SA hydrogel beads crosslinked with $CaCl_2$ solution [37]. Thus, the varying cytotoxic effects of CA may be due to the cell-type specific regulatory mechanisms in response to the level of calcium ion. Osteoblasts are cells that are responsible for bone formation by laying down essential bone matrix proteins, inducing mineralization and regulating osteoclast differentiation [38]. Studies have shown that increasing levels of extracellular Ca^{2+} enhanced the proliferation and/or differentiation of osteoblasts [39, 40]. Varying levels of Ca^{2+} were shown to exert different effects on osteoblasts, in that, 2-4 mM promoted cell proliferation, 6-8 mM modulated OB differentiation, while more than 10 mM was cytotoxic [40]. So, the potential presence of a higher level of Ca^{2+} released from CA into the culture medium, in this study, was apparently not cytotoxic to the primary hAOBs.

In summary, we have shown that the Calcium Alginate/N,O-carboxymethylchitosan hemostatic sponge is cytocompatible to the primary hAOBs, indicating that it could be a promising material for bleeding control in the tooth extraction socket or alveolar bone area. However, *in vivo* and clinical studies regarding its biodegradability and effects on alveolar bone healing would be further required. In addition, future studies are warranted to evaluate the biocompatibility of the material towards primary gingival or periodontal ligament fibroblasts, in order to develop the CA/NOCC hemostatic sponge that could be applicable to all types of oral tissues.

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