Chiang Mai Dental Journal

Original Article

Cytotoxicity of the Calcium Alginate/ *N,O*-carboxymethylchitosan Hemostatic Sponge on Primary Human Gingival Fibroblasts

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Received: June 10, 2021 • Revised: July 24, 2021 • Accepted: August 4, 2021

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Abstract

To date, chitosan-based hemostatic agents have gained increasing interest from their biocompatibility, inexpensiveness and hemostatic capability, especially in coagulopathic conditions. We have developed a functionally improved Calcium Alginate/*N,O*-carboxymethylchitosan (CA/NOCC) hemostatic sponge, shown to be biocompatible and biodegradable.

Objectives: To ensure its safe use with the gingival tissue, this study aimed to evaluate the cytotoxicity of CA/NOCC sponge on primary human gingival fibroblasts (GFs).

Methods: Human GFs were cultured with or without the CA/NOCC sponge. Cell morphology was assessed by scanning electron microscopy. Cell viability and proliferation were determined by MTT assays. The levels of Ca^{2+} released into the culture medium were also measured.

Results: Gingival fibroblasts cultured with the CA/NOCC sponge demonstrated lowered cell density, and significant ultrastructural changes of the cell membrane, by forming numerous blebs and fibrils. From MTT assays, approximately 30% decrease in the proliferation rate was observed. Moreover, the levels of Ca^{2+}, up to 4.6 mM, were detected in the medium of GFs cultured with the CA/NOCC sponge.

Conclusions: It could be implicated that the cytopathic effects on the morphology and proliferative ability of GFs may result from the high level of Ca^{2+} released from the CA/NOCC hemostatic sponge.

Keywords: CA/NOCC sponge, calcium ion, cell morphology, cell proliferation

Introduction

Uncontrolled bleeding is potentially one of the most life-threatening complications in oral surgical procedures, especially for patients with coagulopathic conditions. Reductions in the morbidity and mortality rates, from excessive hemorrhaging, were most likely due to the development and utilization of numerous types of hemostatic agents. However, studies are still ongoing in search for a novel hemostatic material with overall characteristics of biocompatibility, biodegradability, non-immunogenicity, affordability, ease of use, and efficacy in bleeding control in both normal and coagulopathic conditions.(1) To date, Gelfoam® and Surgicel® are still widely used for intraoral bleeding control due to their biocompatibilities, accessibilities, ease of usage and storage.(1,2) Nonetheless, their hemostatic efficiencies, when used in patients

with coagulation problems, were shown to be somewhat limited.[2] In addition, some negative effects were associated with these two hemostatic agents, in that, the acidic properties of Surgicel® may cause inflammation, tissue necrosis, neurological complications and may interfere with bone regeneration. On the other hand, the volumetric swelling property of Gelfoam® may result in excessive compression of vital adjacent structures.[2,3] To overcome these limitations, novel hemostatic materials are actively being developed and tested.

Chitosan (CS) is a natural polymer, derived from N-deacetylation of chitin which is abundantly present in the shells of shrimps and crabs. It has been increasingly accepted as an exceptional biomaterial that is biocompatible and biodegradable with capabilities in microbial control, wound healing support and hemostasis.[4,5] To date, a number of CS-based hemostatic agents have been approved by the Food and Drug Administration of the United States (FDA) and are commercially available, including HemCon® Dental Dressing (HemCon Medical Technologies Inc., Portland, OR, USA), Celox® (MedTrade Products Ltd., Cheshire, UK), and Axiostat® (Axio Biosolutions Private Ltd., Gujarat, India). Most importantly, the effectiveness of CS in bleeding control was shown to be independent of the coagulation pathway.[6] Its hemostatic efficacy in coagulopathic conditions has been tested and verified in a number of *in vivo*,[7,8] and clinical studies.[9-11] Nevertheless, CS is less soluble in water or at neutral pH range, which is potentially the limiting property in developing CS-based materials to support broader biomedical uses. Therefore, water-soluble CS derivatives, with enhanced material characteristics and hemostatic efficiencies, have been prepared and tested.[12-14]

Previously, we have prepared *N,O*-carboxymethylchitosan (NOCC), which is a water-soluble derivative of CS, through the carboxymethylation reaction of chitosan. The negatively-charged material is biocompatible, hydrophilic and shown to promote platelets activation/aggregation, *in vitro* blood coagulation and superior bleeding control over chitosan.[14] With its improved properties, NOCC has been used to develop biomaterials to support wider clinical applications.[12,15,16] The Calcium Alginate/*N,O*-carboxymethylchitosan (CA/NOCC) hemostatic sponge has been previously developed in our lab.[17,18] Through *in vitro*, *in vivo* and clinical investigations, CA/NOCC was shown to be biocompatible and degradable,[17,19] with higher hemostatic capabilities over SPONGOSTAN®.[18,20] Most recently, we have demonstrated that the CA/NOCC sponge is not cytotoxic to primary human alveolar osteoblasts (hAOBs), and that their morphology and osteoblastic phenotype were minimally altered,[21] suggesting that the material could potentially be used for bleeding control in tooth extraction sockets.

To further determine if the CA/NOCC sponge could be safely used in the gingival tissue area, we, therefore, aimed to investigate the biocompatibility of the CA/NOCC sponge to human gingival fibroblasts (GFs). The morphological response of the cells was evaluated by scanning electron microscopy. Cell viability and proliferation rate were determined by MTT assays. In addition, the level of Ca^{2+} released from the CA/NOCC sponge was measured.

Materials and Methods

Preparation of the CA/NOCC sponge

The synthesized *N,O*-carboxymethylchitosan (NOCC) in our lab, based on the previously reported method,[13] was utilized to develop the Calcium Alginate/*N,O*-carboxymethylchitosan (CA/NOCC) sponge through the Na^{+}-Ca^{2+} exchange process between sodium alginate (SA; FMC Biopolymer, Philadelphia, USA) and calcium chloride solution ($CaCl_2$; Fluka, Missouri, USA).[17,18] In brief, 0.5 g NOCC and 2.0 g SA were dissolved in 100 ml deionized water, resulting in the 2.5% (wt./vol) highly viscous aqueous solution, which was poured into molds and lyophilized to form 1 mm-thick sponge-like pads. Next, the water-soluble SA/NOCC sponges were immersed into the 10%wt. aqueous $CaCl_2$ solution, with gentle stirring, for 1 h. to produce the water-insoluble CA/NOCC pads which were then washed with deionized water, freeze-dried, cut into circular pieces (6 and 10 mm in diameters), and sterilized by ethylene oxide gas prior to use.

Primary cell isolations and cell culture

The study protocol received approval from the Ethics Committee of the Faculty of Dentistry and Srinakharinwirot University, Bangkok, Thailand (DENTSWU-EC03/2561 and SWUEC/X-154/2562).

Gingival fibroblasts (GFs) were extracted from the non-inflamed, excess gingival tissue surgically removed

along with the non-carious impacted tooth. The gingival tissue was washed twice in PBS, cut into small pieces and placed in 35x10 mm cell culture dishes (Nunc) with minimal amount of culture medium, i.e. Dulbecco Modified Eagle's Medium (DMEM) high glucose (GE Healthcare, Salt Lake City, UT, USA) supplemented with 10% FBS (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 IU/ml of penicillin, 100 *u*g/ml of streptomycin, and 5 *u*g/ml of amphotericin B (Invitrogen). The culture dishes were placed in a 37°C humidified incubator with 5% CO_2 atmosphere. The culture medium was changed twice a week, and GFs outgrowth from the tissue was constantly monitored. Upon confluency, the cells were subcultured into 100x21 mm cell culture dishes (Nunc) and designated as cell passage number 1. For this study, GFs of passage number 3-8 were used.

Cell morphology

The morphological response and attachment of human GFs to the CA/NOCC sponge were evaluated by scanning electron microscopy, as previously reported.[21] Briefly, the trypsinized GFs were plated, at the density of $6x10^4$ cells/well in 1 ml of culture medium, onto the cover glasses with or without the CA/NOCC sponge (size 10 mm in diameter), in the 24-well cell culture plate (Nunc). The culture plate was placed in the 37°C incubator with 5% CO_2 atmosphere for 72 h. Following a rinse with PBS, the cells with or without the CA/NOCC sponge, were fixed with 2.5% glutaraldehyde in deionized distilled water for 4 h., washed with PBS, subjected to gradient ethanol dehydration (25%-50%-70%-100%), and fixed with hexamethyldisilazane (HDMS) for 15 min at RT. After the samples were dried at RT, the morphology of GFs on the cover glass and associated with surface of the CA/NOCC sponge was evaluated with a scanning electron microscope (JSM-6510LV, JEOL Ltd., Tokyo, Japan).

Cell proliferation assays

Human primary GFs were trypsinized and seeded, at the density of $2x10^4$ cells/ml of culture medium, directly into the wells (control group), or onto the CA/NOCC sponge (size 6 mm in diameter) placed in the wells (experimental group) of a 24-well cell culture plate (Nunc). The culture plate was placed in the 37°C incubator with 5% CO_2 for 24 h. to allow cell attachment. The viability levels of GFs on days 1, 3 and 6 were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assays, as reported previously.[21] Briefly, after the removal of culture medium, 550 μl of the solution mixture between the 5 mg/ml MTT/PBS and serum-free culture medium was added to each well, and the plate was incubated at 37°C with 5% CO_2 for 2 h. Next, the solution in each well was carefully aspirated, and the formed formazan crystals were completely dissolved by adding 500 µl of dimethyl sulfoxide (DMSO; Vivantis Technologies, Selangor Darul Ehsan, Malaysia) and keeping the plate in rocking motion at RT for 30 min. The solution was aliquoted, in triplicates, to a 96-well plate, from which the absorbance at the wavelength of 550 nm was measured with a microplate reader (Asys UVM340; Biochrom, Cambridge, UK).

Ca^{2+} concentration measurement

The culture medium was collected from the cell proliferation and cell morphology assays, described above, where GFs were cultured with or without the CA/NOCC sponge. Additionally, the culture medium incubated with only the CA/NOCC sponge, in the same condition and time points, was also collected. The level of Ca^{2+} release into the culture medium was determined with the Quantichrom Calcium Assay Kit (BioAssay Systems, Hayward, USA) according to the recommended protocol. Five microliters of each sample were aliquoted into the wells of a 96-well plate, following by the addition of 200 μl of reaction mixture. The plate was tapped lightly to mix, incubated at room temperature for 3 min, and subjected to a microplate reader (Asys UVM340; Biochrom, Cambridge, UK), where the absorbance at the wavelength of 612 nm was measured. The levels of Ca^{2+} in the culture medium were calculated from the standard curve generated.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.0.1 for Windows, (GraphPad Software, San Diego, California USA, www.graphpad.com). Normality of the data was evaluated with the Shapiro-Wilk Test. Statistical differences among the groups were determined by Student's t-test, for the cell proliferation data, while One-way ANOVA followed by Tukey's multiple comparison test was used for Ca^{2+} levels analysis. The difference at p value of less than 0.05 was considered statistically significant.

Results

Cell morphology

The control GFs, attached to the cover glass, exhibited flat and fusiform cell morphology with relatively smooth cell surface (Figure 1A-B). However, when seeded onto the CA/NOCC material, most of the cells adhered to the cover glass rather than on the surface of the porous CA/NOCC sponge. In addition, there was a significant reduction in cell density, among which, higher numbers of GFs exhibited rough surfaces from the formation of cellular projections and small blebs, when cultured with the CA/NOCC sponge (Figure 1C-D).

On the porous surface of the CA/NOCC sponge, minimal numbers of round or ovoid-shaped cells were observed. They adhered to the surface of the material by forming short filopodia (red arrows in Figure 2). The GFs associated with the CA/NOCC sponge mainly exhibited rough cell membrane, containing numerous fibrils and moderate numbers of blebs formation (yellow arrowheads in Figure 2).

Cell proliferation

The proliferative capabilities of GFs, cultured in direct contact with the CA/NOCC sponge, were assessed by performing MTT assays on days 1, 3 and 6. The growth pattern and proliferation rate of the cells, relatively to the control, were depicted in the line graphs and bar charts, respectively (Figure 3). When cultured with the CA/NOCC sponge, the viability levels of GFs, at days 3 and 6, were lower than those of the control cells (Figure 3A). In addition, proliferation rates of the cells, determined by slope analyses of the viability levels from day 1-6, from five independent experiments, were demonstrated in the bar chart (Figure 3B). The proliferation rate of GFs cultured with the CA/NOCC sponge was about 70%, which was significantly lower than that of the control (p<0.0001).

Calcium ion levels

The amounts of Ca^{2+} released into the culture medium collected at days 1, 3 and 6 of the cell proliferation assays were demonstrated in Figure 4. In the GFs

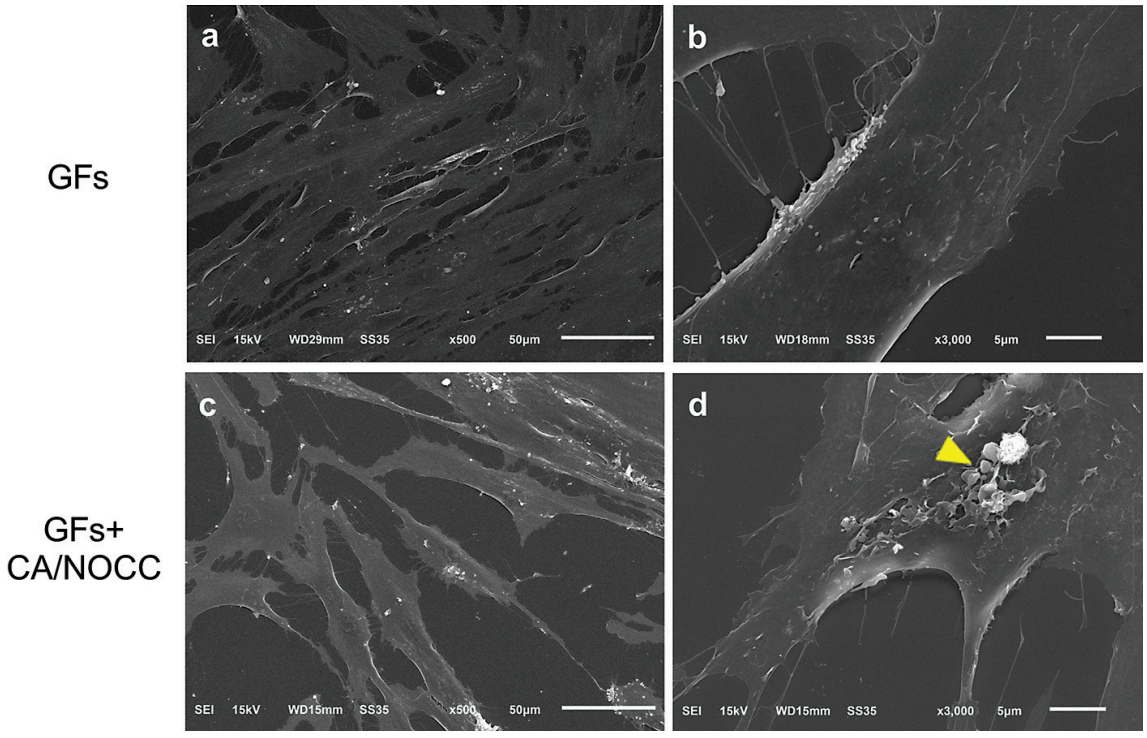

Figure 1: Morphology of human primary gingival fibroblasts (GFs) cultured for 72 h on cover glass (control group) or cover glass with CA/NOCC sponge (study group) and analyzed by scanning electron microscopy (SEM). (A-B) GFs control, (C-D) GFs with CA/NOCC. Formation of blebs on the cell surface was indicated with the yellow arrowhead. Scale bars in A and C = 50 μm; B and D = 5 μm. CA/NOCC, Calcium Alginate/*N,O*-carboxymethylchitosan.

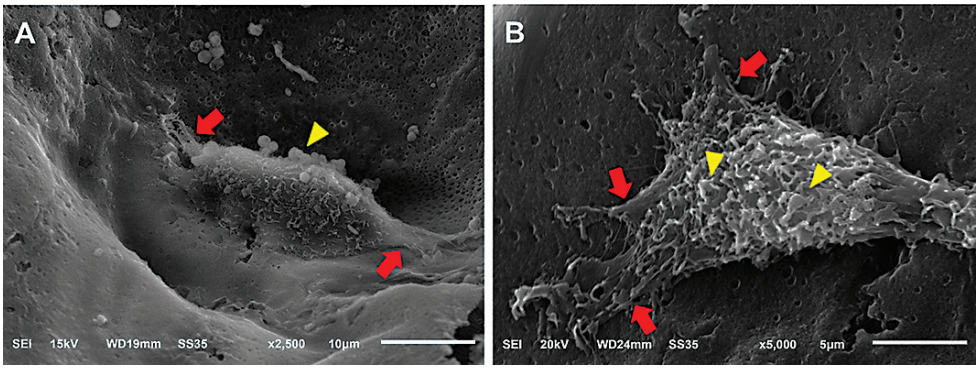

Figure 2: Morphology of gingival fibroblasts (GFs) associated with the surface of CA/NOCC sponge following a 3-day culture and observed by scanning electron microscopy (SEM). Filopodia were indicated with red arrows, while cellular blebs were depicted with yellow arrowheads. Scale bars in A = 10 μm; B = 5 μm. CA/NOCC, Calcium Alginate/*N,O*-carboxymethylchitosan.

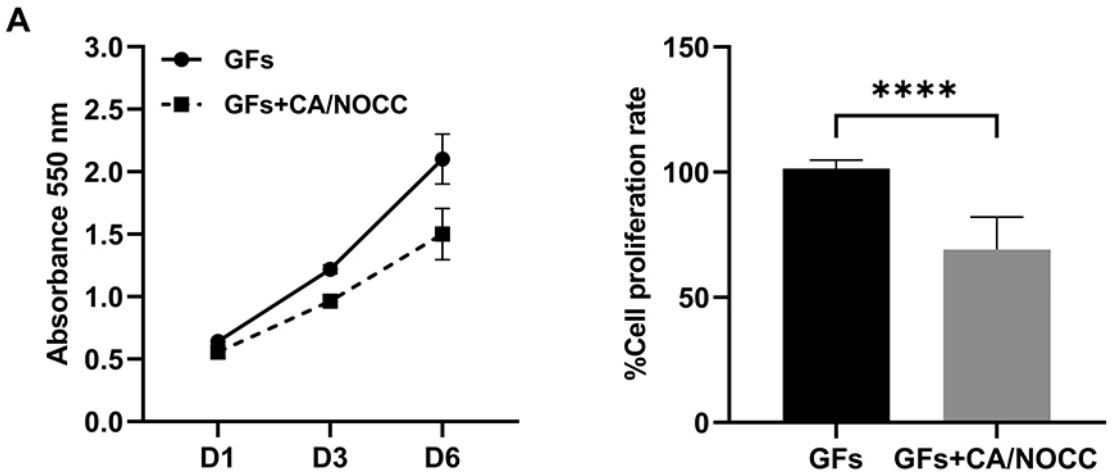

Figure 3: Proliferations of gingival fibroblasts (GFs) were evaluated by MTT assays on days 1, 3 and 6 of cultures with or without the CA/NOCC sponge. (A) The line graph plots demonstrate the viability levels on days (D) 1, 3, and 6. Data are shown as mean absorbance values at 550 nm from one representative experiment, (B) The bar chart demonstrates the proliferation rates of GFs cultured with the CA/NOCC sponge in percentage relative to the control (GFs). Data are shown as means ± SDs from five independent experiments. ****, significantly different from the control group at $p<0.0001$. CA/NOCC, Calcium Alginate/*N,O*-carboxymethylchitosan.

culture, the levels of Ca^{2+} release were relatively stable at all time points (0.27±0.01 mM). When the CA/NOCC sponge was incubated in the culture medium (Medium+-CA/NOCC), the levels of Ca^{2+} in the medium were significantly higher than those of the GFs culture at both days 1 and 3 ($p<0.0001$), demonstrating a clear release of Ca ion from the CA/NOCC sponge into the medium. Interestingly, in the condition where GFs were cultured with the CA/NOCC sponge (GFs+Ca/NOCC), the release of Ca^{2+} into the medium was highest and significantly different from those in the Medium + CA/NOCC incubation (day 1: $p<0.01$, day 3: $p<0.001$) and the GFs culture (day 1 and 3: $p<0.0001$). At day 6, the levels of Ca^{2+} release from the CA/NOCC sponge were significantly reduced to approximately 33% of the day 3 levels, in both the GFs+CA/NOCC culture (33.4±7.8%) and Medium + CA/NOCC incubation (33.0±15.0%) groups, after the fresh medium replacement at day 3. It could, therefore, suggest an occurrence of the high burst-release of Ca^{2+} from the CA/NOCC sponge during the first 3 days of culture or material incubation.

According to the technical documentation, DMEM high glucose (Hyclone) contains approximately 1.36 mM of Ca^{2+} content. The level of Ca^{2+} measured from the prepared cell culture medium was approximately 1.42±0.05 mM. The medium was used for the GFs culture with and

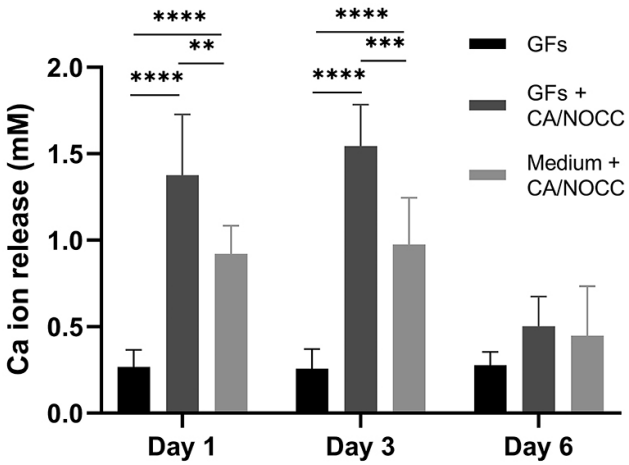

Figure 4: Levels of Ca ion released into the culture medium at Days 1, 3 and 6 of cell proliferation assays. Data are shown as means ± SDs from five independent experiments. **, significantly different at $p<0.01$; ***, significantly different at $p<0.001$; ****, significantly different at $p<0.0001$. GFs, gingival fibroblasts; CA/NOCC, Calcium Alginate/*N,O*-carboxymethylchitosan; Medium, DMEM high glucose supplemented with 10% FBS, 2 mM L-glutamine, 100 IU ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin, and 5 μg ml^{-1} of amphotericin B.

without the CA/NOCC sponge and the incubation of CA/NOCC material. In this study, the 6 mm- and 10 mm-diameter CA/NOCC sponge pads were used for the cell proliferation and cell morphology assays, respectively. The total calcium ion levels (Level of Ca^{2+} released+1.42 mM Ca^{2+} content in the culture medium), in the culture medium at Day 3 of continuous culture/incubation, from the 2 assays are shown in Table 1. In the GFs culture, the levels of Ca^{2+} were relatively comparable and in the range of 1.53-1.68 mM. When the CA/NOCC sponge was incubated in the culture medium (Medium+CA/NOCC), the levels of Ca^{2+} in the medium were significantly increased from those of the GFs culture i.e., approximately 2.40 mM for the 6-mm and 3.69 mM for the 10-mm CA/NOCC sponges, respectively. Interestingly, in the condition where GFs were cultured with the CA/NOCC sponge (GFs+Ca/NOCC), the levels of Ca^{2+} in the medium (6-mm sponge: 2.96 mM, 10-mm sponge: 4.60 mM) were about 1.2 times higher than those from the Medium+CA/NOCC incubation. Moreover, the levels of Ca^{2+} in the conditions using the 10-mm CA/NOCC sponge were about 1.5 times of those using the 6-mm material.

Discussion

To develop a biomaterial with improved hemostatic efficiency, it is essential to select proper components for the preparation. The chitosan derivative, *N,O*-Carboxymethylchitosan (NOCC), was shown to contain numerous beneficial properties, including biocompatibility, biodegradability, wound healing promotion as well as having antimicrobial and hemostatic capabilities.[12,17,18] Alginate is a natural anionic polysaccharide,

Table 1: Total calcium ion levels (mM) from day 3 of GFs culture with or without the CA/NOCC sponge with varying diameters

CA/NOCC sponge diameter	Total Calcium ion levels (mM)		
	GFs + CA/NOCC	Medium + CA/NOCC	GFs
6 mm	2.96 ± 0.24	2.40 ± 0.27	1.68 ± 0.11
10 mm	4.60 ± 0.31	3.69 ± 0.30	1.53 ± 0.22

Values represent means ± SDs from three independent experiments.
GFs, gingival fibroblasts; CA/NOCC, Calcium Alginate/*N,O*-carboxymethylchitosan; Medium, DMEM high glucose supplemented with 10% FBS, 2 mM L-glutamine, 100 IU ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin, and 5 μg ml^{-1} of amphotericin B.

extracted from seaweeds, that has shown to be hydrophilic, biocompatible, biodegradable and wound healing accelerating. Moreover, in the forms of sodium alginate and calcium alginate, they demonstrate hemostatic efficacy.[22,23] From the favorable properties of both materials, we have prepared the CA/NOCC hemostatic sponge from the SA and NOCC solution mixture, which was casted and freeze-dried into a sponge-pad form. The material was then dipped in the $CaCl_2$ solution, in which the Na^+-Ca^{2+} exchange reaction occurred.[17,18] We have previously exhibited that the CA/NOCC sponge was cytocompatible and supported the proliferative ability of the L929 mouse fibroblasts,[17] and the primary hAOBs isolated from the jaw bone.[21]

Unexpectedly, in this study, we have observed that the ultrastructural morphology of human GFs was significantly altered, when the cells were cultured with the CA/NOCC sponge. From SEM analyses, GFs cultured with the material were mainly attached to the underlying cover glass with significant reduction in cell density. Moreover, those GFs on the cover glass and the few material-associated cells exhibited irregularly-textured cell surface with the presence of numerous large fibrils and cellular blebbing. These results were in contrast to our recent report showing that, when the hAOBs were cultured with the CA/NOCC sponge, the cell density was comparable to the control and the cell surface was homogenously covered with small fibrils.[21] In another study by Li *et al.*[24] when human osteoblast cell line (MG63) was cultured with the calcium alginate-chitosan scaffold, enhanced cell density was observed, and the cells attached to the material surface was covered with numerous microvilli.[24] In regards to the morphological alterations observed with GFs, the formation of blebs at the cell surface could be indicative of possible cell necrosis or apoptosis[25,26], while the presence of fibrils was associated to the cells' attempt to remove toxic substances.[27]

The effect of CA/NOCC sponge on the cell proliferation capacity was also assessed by utilizing MTT assay. When the GFs were cultured with the CA/NOCC sponge, their proliferation rate was significantly reduced to about 70% of the control, unlike the minimally-altered proliferative ability of hAOBs, shown in our previous study.[21] Evidently, the cell proliferation result was in coordination with the ultrastructural changes of the cells observed from the SEM analyses, suggesting that the CA/NOCC sponge did exert some cytotoxic effects toward GFs.

To determine the potential causes of the morphological and functional changes of GFs, cultured in direct contact with CA/NOCC sponge, the previously reported cytotoxic effects of NOCC and CA were reviewed. Studies have demonstrated the cytocompatibility of NOCC to various cell types, including mouse fibroblast cell line (NIH3T3),[28] primary human dermal fibroblasts.[29] and breast cancer cells (MCF-7).[30] On the other hand, previous studies have demonstrated that calcium alginate was cytotoxic towards fibroblasts and epidermal cells.[31-34] Suzuki *et al.*[32,33] have tested alginate dressings with varying calcium content, and they have shown that proliferation of L929 fibroblasts was inhibited with increased Ca^{2+} releases. Moreover, studies have shown that Kaltostat®, a CA-hemostatic dressing, induced morphological changes of human embryonic lung fibroblasts (MRC5) and human keratinocytes,[31] and severely impaired the proliferation of L929 murine fibroblasts, partly from the high Ca^{2+} release.[32,33] and possibly from the presence of some bioincompatible contaminants in commercial sodium alginates.[35] In another study by Paddle-Ledinek *et al.*[34], they have exhibited that the extracts of alginate-containing dressings with higher levels of calcium (Algisite M: 4.89 mM, Contreet-H: 3.06 mM and SeaSorb: 3.42 mM), significantly altered the morphology and induced 60-90% loss of proliferative abilities in primary human keratinocytes. In agreement with these reports, we have observed that the proliferative ability of the GFs, cultured with the CA/NOCC sponge, began to deteriorate in association with the exposure of the cells to 2.96±0.24 mM Ca^{2+} content in the medium. Additionally, the cytotoxic changes to the GFs morphology were associated with the level of Ca^{2+} as high as 4.60±0.31 mM, in the culture medium.

Calcium ions (Ca^{2+}) has been shown to be one of the critical components in various phases of the cell cycle. The optimal extracellular Ca^{2+} levels, required for the accumulation of DNA replicators, were shown to vary among different cells types. Keratinocytes and colon cells need around 0.05 to 0.1 mM of extracellular Ca^{2+}, while fibroblasts, hepatocytes and thymic lymphoblasts require approximately 1.0 to 1.5 mM.[36,37] Thus, the 1.42 mM Ca^{2+} level in our prepared culture medium was proper for the growth and functions of human GFs. On the other hand, studies have demonstrated the roles Ca^{2+} in the process of toxic cell death or apoptosis. Enhanced intra-

cellular influx or redistribution of Ca^{2+} among cellular compartments can induce toxic reactions and irreversible injuries to various cells, by impairing their mitochondrial function, disturbing the cytoskeletal organization, and activating Ca^{2+}-involved catabolic processes.[38,39] By using MTT assays, which measure the mitochondrial activity of the cells, we have shown a significant reduction in the proliferation rates of GFs, cultured in the condition with a high level of Ca^{2+} released from the CA/NOCC sponge. In addition, those GFs demonstrated morphological changes by forming numerous protrusions and blebs at the cell surface, which could be indicative of the cytoskeletal alterations resulting from toxic injury to the cells.[39] By measuring the release of Ca^{2+} from the CA/NOCC sponge, in this study, considerably high levels of Ca ion were detected in the culture medium since day 1 of culture and followed by a slight increase at day 3. While, at day 6, we have observed about 67% decrease in the levels of Ca^{2+} release, which could, most likely, be from the fresh culture medium replacement and the almost saturated levels of Ca^{2+} released from the material since day 3. It could be postulated that, the high levels of Ca^{2+} discharged from the CA/NOCC sponge during days 1-3 of culture or incubation, may be mainly caused by the dissolution of $CaCl_2$ residue left on the surface of the material after the material cleaning process with deionized water, rather than the Ca^{2+} and Na^{+} exchange process which would occur during the degradation of the CA/NOCC sponge in the culture medium that was found to take more than 10 weeks.[17] Thus, future studies on a 24 h. pre-washed CA/NOCC sponge, to remove the excess Ca ion, would be planned and evaluated of its cytocompatibility to the GFs.

Interestingly, we have previously observed minimal cytotoxic effects of the CA/NOCC sponge on primary hAOBs.[21] Most likely, it may be due to the cell-type specific regulatory mechanisms in response to the Ca^{2+} concentration. Maeno *et al.*[40] have exhibited that, different extracellular Ca^{2+} concentrations were shown to have varying effects on osteoblasts, where 2-4 mM supported cell proliferation, 6-8 mM regulated cell differentiation, while more than 10 mM was toxic. Therefore, the high level of Ca^{2+} release from the CA/NOCC sponge was considered to be in the safe range in maintaining normal human osteoblast growth and functions while it could be potentially cytotoxic to human gingival fibroblasts.

Conclusions

Our results indicate that the CA/NOCC hemostatic sponge, which released a significantly higher level of Ca^{2+}, demonstrated cytotoxic effects towards human GFs by altering the cell morphology and reducing the cell proliferation rates. Therefore, to develop the CA/NOCC hemostatic sponge that is biocompatible with no negative effect on gingival tissue regeneration, future studies on the pre-washed CA/NOCC sponge, the materials with varying ratios of CA to NOCC, or identification of other potential toxic factors would be warranted.

Acknowledgments

This study was financially supported by the Research Grant of the Faculty of Dentistry, Srinakharinwirot University (Grant number 320/2561) We would like to thank the Department of Oral Stomatology, Faculty of Dentistry, Srinakharinwirot University for their support of the laboratory space and equipment.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Kamoh A, Swantek J. Hemostasis in oral surgery. Dent Clin North Am. 2012;56(1):17-23.
2. Kumar S. Local hemostatic agents in the management of bleeding in oral surgery. Asian J Pharm Clin Res. 2016;9(3):35-41.
3. Brodbelt AR, Miles JB, Foy PM, Broome JC. Intraspinal oxidised cellulose (Surgicel) causing delayed paraplegia after thoracotomy--a report of three cases. Ann R Coll Surg Engl. 2002;84(2):97-9.
4. Cicciù M, Fiorillo L, Cervino G. Chitosan use in Dentistry: a systematic review of recent clinical studies. Mar Drugs. 2019;17(7):417.
5. Khan MA, Mujahid M. A review on recent advances in chitosan based composite for hemostatic dressings. Int J Biol Macromol. 2019;124:138-47.
6. Chen KY, Chen YC, Lin TH, Yang CY, Kuo YW, Lei U. Hemostatic enhancement via chitosan is independent of classical clotting pathways—a quantitative study. Polymers. 2020;12(10):2391.
7. Koksal O, Ozdemir F, Cam Etoz B, Buyukcoskun NI, Sigirli D. Hemostatic effect of a chitosan linear polymer (Celox®) in a severe femoral artery bleeding rat model under hypothermia or warfarin therapy. Ulus Travma Acil Cerrahi Derg. 2011;17(3):199-204.

8. Millner RW, Lockhart AS, Marr R, Jones K. Omni-Stat® (Chitosan) arrests bleeding in heparinised subjects *in vivo*: an experimental study in a model of major peripheral vascular injury. Eur J Cardiothorac Surg. 2011;39(6):952-4.
9. Kumar KR, Kumar J, Sarvagna J, Gadde P, Chikkaboriah S. Hemostasis and post-operative care of oral surgical wounds by hemcon dental dressing in patients on oral anticoagulant therapy: a split mouth randomized controlled clinical trial. J Clin Diagn Res. 2016;10(9):Zc37-40.
10. Sharma S, Kale TP, Balihallimath LJ, Motimath A. Evaluating effectiveness of axiostat hemostatic material in achieving hemostasis and healing of extraction wounds in patients on oral antiplatelet drugs. J Contemp Dent Pract. 2017;18(9):802-6.
11. Pippi R, Santoro M, Cafolla A. The use of a chitosan-derived hemostatic agent for postextraction bleeding control in patients on antiplatelet treatment. J Oral Maxillofac Surg. 2017;75(6):1118-23.
12. Shariatinia Z. Carboxymethyl chitosan: properties and biomedical applications. Int J Biol Macromol. 2018; 120:1406-19.
13. Janvikul W, Thavornyutikarn B. New route to the preparation of carboxymethylchitosan hydrogels. J Appl Polym Sci. 2003;90:4016-20.
14. Janvikul W, Uppanan P, Thavornyutikarn B, Krewraing J, Prateepasen R. *In vitro* comparative hemostatic studies of chitin, chitosan, and their derivatives. J Appl Polym Sci. 2006;102(1):445-51.
15. Liu L, Lv Q, Zhang Q, Zhu H, Liu W, Deng G, *et al*. Preparation of carboxymethyl chitosan microspheres and their application in hemostasis. Disaster Med Public Health Prep. 2017;11(6):660-7.
16. Zhong QK, Wu ZY, Qin YQ, Hu Z, Li SD, Yang ZM, *et al*. Preparation and properties of carboxymethyl chitosan/alginate/tranexamic acid composite films. Membranes. 2019;9(1):11.
17. Janvikul W, Uppanan P, Thavornyutikarn B. Calcium alginate, *N,O*-carboxymethylchitosan and their blends: *In vitro* biocompatibility and biodegradability. Proceedings of The World Congress on Bioengineering 2007 (WACBE); 2007, July 9-11; Bangkok, Thailand.
18. Janvikul W, Uppanan P, Kosorn W, Phusuksombati D, Prateepasen R. Evaluation of efficacy of chitosan derivative based hemostat: *In vitro* and *in vivo* studies. Proceedings of The 2nd International Symposium on Biomedical Engineering (ISBME 2006); 2006, November 8-10; Bangkok, Thailand.
19. Thavornyutikarn B, Kosorn W, Surattanawanich P, Wongparami U, Janvikul W. Chitosan derivative-based hemostat: Irritation, hypersensitivity and absorbability study. J Met Mater Miner. 2013;23:49-52.
20. Janvikul W, Thavornyutikarn B, Kosorn W, Surattanawanich P. Clinical study of chitosan-derivative-based hemostat in the treatment of split-thickness donor sites. Maejo Int J Sci Technol. 2013;7:385-95.
21. Phaewphala K, Kosorn W, Janvikul W, Kitrueangphatchara K, Sricholpech M. Biocompatibility of a chitosan-derived hemostatic agent with human alveolar osteoblasts. M Dent J. 2020;40(3):277-88.
22. Taskin AK, Yasar M, Ozaydin I, Kaya B, Bat O, Ankarali S, *et al.* The hemostatic effect of calcium alginate in experimental splenic injury model. Ulus Travma Acil Cerrahi Derg. 2013;19(3):195-9.
23. Szekalska M, Puciłowska A, Szymańska E, Ciosek P, Winnicka K. Alginate: current use and future perspectives in pharmaceutical and biomedical applications. Int J Polym Sci. 2016; Article ID 7697031. doi:10.1155/2016/7697031
24. Li Z, Ramay HR, Hauch KD, Xiao D, Zhang M. Chitosan-alginate hybrid scaffolds for bone tissue engineering. Biomaterials. 2005;26(18):3919-28.
25. Barros LF, Kanaseki T, Sabirov R, Morishima S, Castro J, Bittner CX, *et al.* Apoptotic and necrotic blebs in epithelial cells display similar neck diameters but different kinase dependency. Cell Death Differ. 2003;10(6):687-97.
26. Mills JC, Stone NL, Erhardt J, Pittman RN. Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. J Cell Biol. 1998;140(3):627-36.
27. Messer RL, Bishop S, Lucas LC. Effects of metallic ion toxicity on human gingival fibroblasts morphology. Biomaterials. 1999;20(18):1647-57.
28. Li X, Kong X, Zhang Z, Nan K, Li LL, Wang XH, *et al*. Cytotoxicity and biocompatibility evaluation of *N,O*-carboxymethyl chitosan/oxidized alginate hydrogel for drug delivery application. Int J Biol Macromol. 2012;50(5):1299-305.
29. Rasad MSBA, Halim AS, Hashim K, Rashid AHA, Yusof N, Shamsuddin S. *In vitro* evaluation of novel chitosan derivatives sheet and paste cytocompatibility on human dermal fibroblasts. Carbohydr Polym. 2010;79(4):1094-100.
30. Anitha A, Rani VD, Krishna R, Sreeja V, Selvamurugan N, Nair SV, *et al*. Synthesis, characterization, cytotoxicity and antibacterial studies of chitosan, O-carboxymethyl and *N,O*-carboxymethyl chitosan nanoparticles. Carbohydr Polym. 2009;78(4):672-7.
31. Rosdy M, Clauss LC. Cytotoxicity testing of wound dressings using normal human keratinocytes in culture. J Biomed Mater Res. 1990;24(3):363-77.
32. Suzuki Y, Nishimura Y, Tanihara M, Suzuki K, Kitahara AK, Yamawaki Y, *et al.* Development of alginate gel dressing. J Artif Organs. 1998;1(1):28-32.
33. Suzuki Y, Nishimura Y, Tanihara M, Suzuki K, Nakamura T, Shimizu Y, *et al.* Evaluation of a novel alginate gel dressing: cytotoxicity to fibroblasts *in vitro* and foreign-body reaction in pig skin *in vivo.* J Biomed Mater Res. 1998;39(2):317-22.

34. Paddle-Ledinek JE, Nasa Z, Cleland HJ. Effect of different wound dressings on cell viability and proliferation. Plast Reconstr Surg. 2006;117(7 Suppl):110S-8S.
35. Grasdalen H, Larsen B, Smisrod O. 13C-NMR studies of monomeric composition and sequence in alginate. Carbohydr Res. 1981;89(2):179-91.
36. Whitfield JF, Bird RP, Chakravarthy BR, Isaacs RJ, Morley P. Calcium-cell cycle regulator, differentiator, killer, chemopreventor, and maybe, tumor promoter. J Cell Biochem Suppl. 1995;22:74-91.
37. Kulesz-Martin MF, Fabian D, Bertram JS. Differential calcium requirements for growth of mouse skin epithelial and fibroblast cells. Cell Tissue Kinet. 1984;17(5):525-33.
38. Sank A, Chi M, Shima T, Reich R, Martin GR. Increased calcium levels alter cellular and molecular events in wound healing. Surgery. 1989;106(6):1141-7.
39. Orrenius S, McCabe MJ Jr., Nicotera P. Ca(2+)-dependent mechanisms of cytotoxicity and programmed cell death. Toxicol Lett. 1992;64-65:357-64.
40. Maeno S, Niki Y, Matsumoto H, Morioka H, Yatabe T, Funayama A, *et al*. The effect of calcium ion concentration on osteoblast viability, proliferation and differentiation in monolayer and 3D culture. Biomaterials. 2005;26(23):4847-55.