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RESEARCH ARTICLE

Effect of chitosan–hydroxyapatite scaffolds seeded with cryopreserved hADMSCs on bone sialoprotein secretion

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ABSTRACT

Tooth extraction can lead to alveolar bone resorption, requiring regenerative approaches using biomaterial scaffolds combined with stem cells. Chitosan–hydroxyapatite (CS–HA), a well-established scaffold that mimics the composition of human bone, combined with stem cells represents a promising strategy to promote bone formation. This study aimed to evaluate the effect of chitosan–hydroxyapatite (CS-HA) scaffolds seeded with cryopreserved human adipose-derived mesenchymal stem cells (hADMSCs) on bone sialoprotein (BSP) secretion. The hADMSCs used in this study were commercially obtained cryopreserved cells (ATCC® PCS-500-011™) and were characterized by flow cytometry. Scaffolds were fabricated using a freeze-drying method by combining chitosan and hydroxyapatite in a 1 : 1 ratio, followed by freezing at −80 °C. A post-test-only control group design was employed, consisting of 36 samples divided into three groups: positive control (CS-HA + hADMSCs + a-MEM), negative control (hADMSCs + osteogenic medium), and treatment (CS-HA + hADMSCs + osteogenic medium), and BSP levels were subsequently quantified on days 7, 14, 21, and 28 using ELISA. One-way ANOVA demonstrated a significant difference among groups ($p = 0.000$), with the highest BSP secretion observed in treatment group on day 14 (BSP levels 44.29 ± 2.58), followed by treatment group on day 28 (BSP levels 46.19 ± 7.64). The significantly elevated BSP secretion in the treatment group on day 14 demonstrates osteoinductive characteristics of the CS-HA scaffold, supporting its potential application in bone tissue engineering and regeneration.

Keywords: bone sialoprotein; bone remodelling; chitosan hydroxyapatite scaffold; human adipose mesenchymal stem cell

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INTRODUCTION

Bone damage caused by tumors, trauma, and genetic factors can lead to serious aesthetic problems and tissue dysfunction. Tooth loss due to tooth extraction can result in bone damage, such as a decrease in alveolar bone volume, which affects long-term healing.[1,2] Bone tissue can naturally regenerate, allowing minor bone damage to heal without medical intervention. However, if bone damage exceeds a 50% reduction in bone circumference, it can lead to complications such as malunion, nonunion, and pathological fractures.[3] As a result, severe bone damage requires medical intervention, such as bone grafting or bone transplantation.[4]

Bone tissue engineering is a biological engineering method to restore and recover bone tissue function by integrating engineering principles, including cells, scaffolds, and regulatory signals.[5] Bone grafts promote bone regeneration through three main mechanisms: an osteoinductive effect, inducing the differentiation of osteoprogenitor cells into cell lineages; an osteoconductive effect, acting as a temporary scaffold to induce osteoblast proliferation and angiogenesis; and an osteogenic effect, whereby bone graft materials are required to be biocompatible to preserve cell viability.[6,7]

Scaffold biocompatibility plays an important role in facilitating tissue integration and regeneration.[8] Scaffolds have microstructures and

porosity that function as extracellular matrices (ECM), providing chemical and mechanical signaling to regulate cells, cell migration, and support tissues. Interconnected porous structures in scaffolds facilitate cell growth and temporarily support new tissue regeneration. In addition to providing mechanical strength, scaffolds promote chemical protein signaling or growth factors and nutrient transport.[9,10]

Scaffolds composed of chitosan (CH) and hydroxyapatite (HA) polymers have been widely used for bone defect repair due to their biocompatibility properties that closely resemble natural bone structure. CH is a natural polysaccharide derived from the partial deacetylation of chitin found in seashells, shrimp, and crab shells. It has antibacterial properties ideal for wound healing and other tissue engineering applications. In antimicrobial activity, positively charged chitosan binds to cellular DNA, inhibiting microbial RNA production and resulting in microbial damage.[11–13] HA is a bioceramic used in bone repair and regeneration due to its excellent biocompatibility, bioresorbability, and bioactivity.[14] There are two mechanisms of interaction between chitosan and hydroxyapatite (HA), including (a) hydrogen bonding between the amino and hydroxyl groups of chitosan and the hydroxyl ions on the surface of HA, and (b) covalent bonding between the amino groups of chitosan and calcium hydroxyapatite. Combining HA and CH can produce a scaffold with high porosity, which is ideal for bone regeneration.[15,16]

Multipotent mesenchymal stem cells (MSCs) have been identified as potential stem cells found in organs and various tissues throughout the body, including adipose tissue.[17] Human Adipose-Derived Mesenchymal Stem Cells (hADMSCs) are of particular interest due to their high proliferation capacity, minimal invasiveness during harvesting, and abundance. The availability of hADMSCs using cryopreservation techniques in liquid nitrogen (LN_2) aims to maintain cell survival by slowing down the metabolic process, thereby allowing cells to be stored for longer periods.[18,19]

Bone sialoprotein (BSP) is a key marker of bone remodeling and is associated with increased bone mineral density. BSP is the major non-collageneous component of the extracellular matrix (ECM) extensively secreted by bone cells, primarily odontoblasts.[20] BSP forms complexes with HA and triggers HA nucleation to promote bone formation and enhance osteoblast differentiation through the vitronectin receptor (αvβ3).[21,22] Previous studies have shown that bone sialoprotein (BSP) is predominantly secreted by osteoblasts and osteocytes, with smaller amounts produced by osteoclasts, and that BSP levels peak on day 2.[23] However, those studies did not utilize Human Adipose-Derived Mesenchymal Stem Cells (hADMSCs) as the cell source to induce BSP secretion in chitosan–hydroxyapatite scaffolds. Meanwhile, studies on scaffolds have reported that combining chitosan with hydroxyapatite results in good biocompatibility with mesenchymal cells, as indicated by increased cell proliferation within the scaffold.[24]

Therefore, this study focuses on the bone remodeling process using a chitosan–hydroxyapatite (CS–HA) scaffold seeded with cryopreserved hADMSCs, with particular emphasis on evaluating BSP secretion. Increased BSP levels are expected to reflect enhanced osteogenic activity and bone mineralization mediated by osteoblast differentiation.Therefore, study focuses on the bone remodelling process using a chitosan–hydroxyapatite (CS-HA) scaffold seeded with cryopreserved hADMSCs to observe BSP secretion. BSP levels increase as osteoblast-mediated bone mineralization progresses.

MATERIALS AND METHODS

Ethical approval for this study was obtained from the Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga (Number: 0713/HRECC. FODM/VII/2024). This study was a laboratory-based experimental analytic study with post-test only control group design. The samples consisted of cryopreserved hADMSCs (ATCC® PCS-500-

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011™) seeded in CS-HA scaffolds and evaluated for bone sialoprotein secretion.

This study consisted of three research groups, including a negative control group (hADMSCs + osteogenic medium), a positive control (CS-HA scaffolds + hADMSCs + α-MEM medium), and a treatment group (CS-HA scaffolds + hADMSCs + osteogenic medium), each groups consisted of three samples and were observed on days 7, 14, 21, and 28.

Cryopreserved hADMSCs were retrieved from liquid nitrogen storage, then thawed by immersing cryovials in a 37 °C water bath for 2-3 minutes. Cells were transferred to centrifuge tubes, mixed with α-MEM (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2 mM L-glutamine, and centrifuged at 1600 rpm for 5 minutes. The cell pellet was resuspended in fresh supplemented α-MEM and cultured in a T-75 flask at 37 °C with 5% CO_2. Cell characterization was performed at passage 3 using flow cytometry with the Human MSC Analysis Kit (BD Stemflow™) following trypsinization, PBS washing, fixation with 10% formaldehyde for 10 minutes, and incubation with primary antibodies (CD73, CD90, CD105), and a negative cocktail (CD44) for 40 minutes. Data acquisition and the cells were analyzed using a FACS Calibur (BD Biosciences).[25,26]

CS-HA scaffolds were prepared by dissolving 200 mg of medium molecular-weight chitosan (Sigma-Aldrich, St. Louis, MO, USA) in 5 ml of ethanoic acid and stirring for 15 minutes at room temperature. Then 5 ml of sodium hydroxide solution was added to neutralize the solution and form a chitosan gel. The gel was mixed with 200 mg HA powder dissolved in 10 ml of distilled water (aquadest) and stirred until homogeneous. The mixture was centrifuged at 1,500 rpm for 10 minutes and poured into custom molds (5×5×2 mm). The gel was frozen at −80 °C for 2 hours and subsequently freeze-dried for 48 hours. Scaffold morphology, pore structure, and surface characteristics were evaluated using Scanning Electron Microscopy (SEM).[27]

Cell seeding was performed using hADMSCs at passage 5. CS-HA scaffolds were placed in a 36-well plate and seeded with 3 × 10^4 hADMSCs in 500 µL α-MEM, then incubated for 24 hours to allow cell attachment, followed by the addition of 100 µL osteogenic medium containing α-MEM, 10% FBS, 1% penicillin/streptomycin, 50 µg/mL ascorbic acid-2-phosphate, 10 mM β-glycerophosphate, and 10 nM dexamethasone.[28]

BSP secretion was quantified using a human BSP enzyme-linked immunosorbent assay (ELISA) kit (BT LAB) based on a sandwich-assay protocol. Supernatants were collected and analyzed using an ELISA assay to quantify BSP levels. Cells were centrifuged at 2000–3000 rpm for 5 minutes, and the supernatants were prepared at room temperature. ELISA plates were loaded with 100 µL of standards and samples per well and incubated for 90 minutes at 37 °C, followed by aspiration and washing. Biotinylated detection antibody was added and incubated for 60 minutes at 37 °C, washed, then horseradish peroxidase **(**HRP) conjugate (100 µL) was added and incubated for 30 minutes. After five additional washes, 100 µL substrate solution was added and incubated for 10–20 minutes in the dark. The reaction was stopped with 50 µL stop solution, and optical density was measured at 450 nm within 10 minutes using a microplate reader.

Data were analyzed using SPSS v.25. Normality was assessed using the Shapiro–Wilk test, and homogeneity of variance was analyzed using Levene's test. One-way ANOVA was performed followed by Tukey post-hoc test. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

This study consisted of three groups, a negative control group, a positive control group, and a treatment group. Descriptive analysis was performed using the mean and standard deviation to summarize BSP secretion levels. Comparisons were conducted to identify differences among groups. Each group consisted of three samples at each observation time point (days 7, 14, 21, and 28).

Table 1. Results of optical density readings of bone sialoprotein secretion

7th day			14th day			21th day			28th day		
NC1	PC1	T1	NC2	PC2	T2	NC3	PC3	T3	NC4	PC4	T4
18.57	24.29	2929	26.43	17.14	41.43	35.71	42.86	29.29	38.57	40.71	55.00
25.00	27.86	30.00	27.86	23.57	46.43	33.57	45.00	30.71	41.43	42.86	42.14
29.29	29.29	32.86	20.00	34.29	45.00	42.14	42.14	40.00	35.00	45.71	41.43

BSP secretion levels among experimental groups.
NC : negative control (cryopreserved hADMSCs + osteogenic medium);
PC : positive control (CS-HA + cryopreserved hADMSCs + α-MEM);
T : treatment (CS-HA + cryopreserved hADMSCs + osteogenic medium);
1 : day 7
2 : day 14
3 : day 21
4 : day 28

Table 2. Mean and standard deviation of BSP secretion of each group on days 7, 14, 21, and 28

Observation time (day)	Groups	Mean ± SD
7	NC1	24.29 ± 5.39
	PC1	27.14 ± 2.58
	T1	30.71 ± 1.89
14	NC2	24.76 ± 4.19
	PC2	25.00 ± 8.66
	T2	44.29 ± 2.58
21	NC3	37.14 ± 4.46
	PC3	43.33 ± 1.49
	T3	33.33 ± 5.82
28	NC4	38.33 ± 3.22
	PC4	43.10 ± 2.51
	T4	46.19 ± 7.64

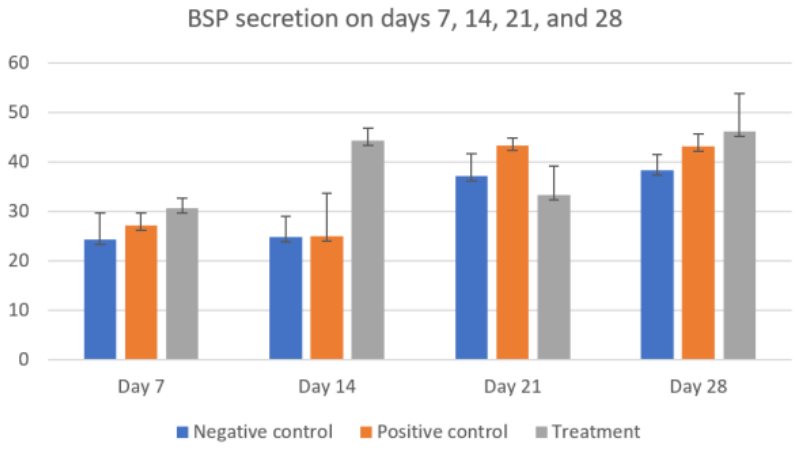

Figure 1. Mean and standard deviation of BSP secretion between study groups on days 7, 14, 21, and 28

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Table 3. Tukey-HSD test result

	NC1	PC1	T1	NC2	PC2	T2	NC3	PC3	T3	NC4	PC4	T4
NC1						*		*		*	*	*
PC1						*		*			*	*
T1												*
NC2						*		*			*	*
PC2						*		*			*	*

(*) indicates a significant difference ($p < 0.05$)

The shapiro-wilk normality test showed that all control and treatment groups had p-values greater than 0.05, indicating that the data were normally distributed. Homogeneity of variance testing using Levene's test on all groups also showed p-values greater than 0.05, indicating that the data between groups were homogeneous and qualified for one-way ANOVA test. The results of one-way anova test revealed a p value of 0.00 ($p < 0.05$), indicating a significant difference between BSP secretion levels among the research groups. Therefore, significant differences can be further analyzed with a Post Hoc Test using Tukey-HSD.

The results showed no significant differences in BSP secretion between the positive and negative control groups on days 7, 14, 21, and 28. In contrast, the highest BSP secretion was observed in the treatment group on day 14.

Tukey HSD analysis in Table 3 showed significant differences in BSP secretion ($p < 0.05$) in groups T2, PC3, PC4, and T4 compared with groups NC1, PC1, NC2, and PC2. Additionally, significant differences were observed between groups NC4 and NC1, as well as between groups T4 and T1.

DISCUSSION

Chitosan–hydroxyapatite (CS-HA) scaffolds have osteoinductive and bioactive properties, along with favorable scaffold biocompatibility and mechanical strength, which support bone remodeling processes.[29] The combination of biomaterials with mesenchymal cells are proven to be effective in stimulating bone regeneration. This finding corresponds with tissue engineering theory, which highlights three components, including stem cells, extracellular matrix scaffolds, and growth factors to stimulate bone growth.[30,31]

In this study, cryopreserved hADMSCs stored in liquid nitrogen containing a cryoprotective agent were thawed using a cell thawing procedure in the first passage. This study utilized cryopreserved hADMSCs because the cryopreservation technique is more cost-effective, time-efficient, and labor-efficient in achieving high cell viability, while maintaining the same potential as freshly cultured cells. This result is supported with a study conducted by Dave et al., which found that there was no significant difference between the use of cryopreserved MSCs and freshly cultured MSCs.[32]

Bone sialoprotein is an extracellular matrix protein found in mineralized tissues such as bone, cartilage, and teeth. BSP has been demonstrated to enhance functions of both osteogenic cells and osteoclastic cells which are important in bone healing. It has been shown to influence key cellular processes, such as proliferation, apoptosis, adhesion, migration, angiogenesis, and extracellular matrix remodeling.[33–35]

Based on the Tukey-HSD test, no significant difference was found between negative control group and positive control group on days 7, 14, 21, and 28. The negative control group consisted of hADMSCs cultured in osteogenic medium without CS-HA scaffold, while the positive control group consisted of CS-HA scaffold seeded with hADMSCs in α-MEM medium. This may be due to the effect of medium used. A study by Luo et al. stated that the use of osteogenic medium can directly increase the differentiation ability

of hADMSCs due to osteogenic supplements, such as dexamethasone, ascorbic acid, and β-glycerophosphate, while α-MEM medium is a basic cell medium without osteogenic supplements. This indicates that despite using α-MEM medium, the CS-HA scaffold is capable of promoting the transformation of mesenchymal cells into osteoblasts.[17,36,37]

The low BSP levels at day 7 likely reflect an early commitment phase, in which hADMSCs were adapting to the microenvironment and beginning lineage specification. At this stage, hADMSCs began to differentiate into pre-osteoblasts in response to stimulation from the scaffold, resulting in detectable but still modest BSP secretion. Nugraha et al. noted that osteocalcin, BSP, and osteopontin are mainly secreted by mature osteoblasts at the next stage of differentiation as markers of bone mineralization.[38] However, BSP secretion on day 14 showed a significant difference between the NC2 and T2 groups. The pronounced rise in BSP at day 14 particularly in the treatment group was consistent with active pre-osteoblast to immature osteoblast transition and initial ECM production, when BSP secretion peaks to nucleate mineral deposition.[39] This finding aligns with a research by Kamadjaja et al., which showed that the CS-HA scaffold stimulates the differentiation of mesenchymal cells into osteoblasts and promotes markers of bone matrix formation.[23] A significant increase in BSP levels was also observed in the P2 group. This may occur due to CS-HA scaffold, which is expected to increase BSP secretion before the BSP peak on day 21. The subsequent decrease observed in the P3 group may reflect the peak of BSP secretion on day 14, followed by a natural decline in the following periods. Such a post-peak reduction likely corresponds to progression from a BSP-rich matrix production phase toward active mineral deposition and matrix maturation, during which BSP synthesis may be downregulated or the protein becomes incorporated into the mineralized matrix and is therefore less detectable in the culture supernatant. In addition, this probably occurred due to cell density and environment affecting osteogenic capacity. Research by Yazid et al. explained that cell seeding density can affect cell proliferation, differentiation, and cell interaction.[40] Despite this decline, the persistence of BSP secretion at day 28 may represent continued matrix mineralization processes, even though peak biosynthesis has likely passed. According to Nugraha et al., the peak of BSP secretion occurs on day 21. However, BSP secretion in this study was still detectable up to day 28 and had the highest average value among all research groups.[24]

In summary, the BSP secretion pattern observed across 28 days indicates that CS–HA scaffolds effectively guide the osteogenic differentiation of cryopreserved hADMSCs. The scaffold's bioactive properties partially replace the need for osteogenic supplements, demonstrating substantial osteoinductive potential. Cryopreserved hADMSCs responded consistently to these treatments, confirming their suitability for scaffold-based regenerative strategies. Collectively, these findings support the application of CS–HA scaffolds seeded with cryopreserved hADMSCs as a potentially promising approach for bone tissue engineering, capable of sustaining osteogenic activity and promoting matrix maturation over extended culture periods.

CONCLUSION

This study demonstrates that CS-HA scaffolds seeded with cryopreserved hADMSCs significantly influence bone sialoprotein (BSP) secretion, indicating active osteogenic activity and extracellular matrix mineral maturation. The temporal pattern of BSP expression, with a peak observed on day 14 followed by a decline, reflects a natural progression of matrix development rather than reduced cellular function. These findings highlight the potential of CS-HA scaffolds combined with hADMSCs as a promising strategy to support bone regeneration. Future research integrating additional osteogenic biomarkers such as OCN, RUNX2, and ALP is warranted to broaden understanding of the regenerative mechanism and validate the therapeutic potential of this scaffold–cell system.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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