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Chitosan–gelatin scaffold reinforced with carbonate hydroxyapatite enhances bone healing in rat extraction sockets: An immunohistochemical study

Devi Rianti [a,*], Bunga Fauzia [b,c], Taufan Bramantoro [d], Rini Devijanti Ridwan [e], Asti Meizarini [a], Sisca Meida Wati [f], Wibi Riawan [g], Ardiyansyah Syahrom [h], Meircurius Dwi Condro Surboyo [i]

[a] Departement of Dental Material, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, 60132, Indonesia
[b] Magister Dental Science Program, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, 60132, Indonesia
[c] Departement of Dental Materials, Faculty of Dentistry, Hang Tuah University, Surabaya, 60111, Indonesia
[d] Departement of Dental Public Health, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, 60132, Indonesia
[e] Departement of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, 60132, Indonesia
[f] Departement of Oral and Maxillofacial Pathology, Faculty of Dental Medicine, Airlangga University, Surabaya, 60132, Indonesia
[g] Department of Biochemistry and Molecular, Laboratory of Biochemistry and Biomolecular, Faculty of Medicine, Brawijaya University, Malang, 65145, Indonesia
[h] Medical Devices and Technology Centre (MEDITEC), Institute of Human Centered qnd Engineering (iHumEn), Universiti Teknologi Malaysia, UTM, Skudai, Johor, 81310, Malaysia
[i] Departement of Oral Medicine, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, 60132, Indonesia

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ABSTRACT

Background: Tooth extraction commonly leads to alveolar bone loss, which can compromise future prosthetic rehabilitation. Biomaterial-based socket preservation aims to support hard-tissue healing and stabilize the extraction site.

Objective: To assess the effect of a chitosan–gelatin scaffold reinforced with carbonate hydroxyapatite (CG-CHA) on bone healing in Wistar rats extraction sockets by evaluating immunohistochemical expression of Bone morpogenetic protein 2 (BMP-2), Runt-related transcription factor 2 (RUNX2) and Receptor activator of nuclear κB (RANK).

Materials and methods: A true-experimental randomized post-test only control group design was used with 24 male Wistar rats. Following maxillary molar extraction, rats were assigned to either a control group (extraction only) or a CG-CHA scaffold group (n = 12 each). Mandibles were collected on days 7, 14, and 21 (n = 4 per time point per group). Immunohistochemistry were performed to evaluate the expression of BMP-2, RUNX2, and RANK. Data were analyzed using independent t-tests with significance at $p < 0.05$.

Results: The CG-CHA group showed greater bone formation and significantly higher BMP-2, RUNX2, and RANK expression at days 7, 14, and 21 compared to controls ($p < 0.05$).

Conclusion: Placement of a CG-CHA scaffold in extraction sockets improved bone healing and increased osteogenic marker expression in rats. These findings indicate that CG-CHA supports favorable socket healing and may be useful for alveolar bone preservation strategies.

1. Introduction

Tooth extraction remains a common dental procedure and is frequently followed by physiological alveolar bone resorption, which can compromise future prosthetic and esthetic outcomes.[1,2] Alveolar ridge reduction may exceed 25% within the first year and continue to 40–60% over three years, with dimensional losses of approximately 3.9 mm horizontally and 1.7 mm vertically in the first three months.[3,4] Healing of the extraction socket involves sequential hemostatic, inflammatory, proliferative, and remodeling phases, and insufficient support during these phases may lead to incomplete regeneration and structural collapse.[5,6]

* Corresponding author. Department of Dental Materials, Faculty of Dental Medicine, Universitas Airlangga, Jalan Prof. Dr. Moestopo Street 47, Surabaya, 60132, Indonesia.
E-mail address: devi-r@fkg.unair.ac.id (D. Rianti).

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Alveolar ridge preservation procedures have been introduced to minimize post-extraction bone loss.[7] Scaffolds serve as temporary extracellular matrices that help stabilize the healing site, maintain volume, and support cell attachment, proliferation, and differentiation.[8–11] An ideal scaffold should be biocompatible, bioresorbable, osteoconductive, mechanically stable, and possess a porous structure conducive to cell migration and nutrient exchange.[12,13] Single-component scaffolds tend to have limitations in mechanical strength and biological performance, therefore composite materials combining organic and inorganic components are increasingly utilized.[14,15]

Chitosan, gelatin, and carbonate-substituted hydroxyapatite (CHA) represent promising biomaterials in bone tissue engineering.[12,16] Gelatin contains cell-adhesive motifs and supports biocompatibility and biodegradation, while chitosan contributes to structural integrity and bioactivity.[17–19] CHA offers osteoconductive and biocompatible properties that closely resemble natural bone mineral and demonstrates superior remodeling characteristics compared with non-substituted hydroxyapatite.[20,21] Previous in vitro work has shown that a 30:70 (w/w) chitosan–gelatin to CHA ratio yields optimal porosity and mechanical strength for osteoblast growth, with pore sizes in the range of 200–350 μm.[10]

Although promising in vitro evidence exists, in vivo validation is necessary to evaluate biological performance during socket healing. The inflammatory, proliferative, and remodeling stages of bone repair typically occur within the first three weeks following extraction, making this period critical for scaffold evaluation. Previous studies on CG-CHA have primarily emphasized general bone regeneration outcomes, while the temporal expression of key osteogenic and remodeling markers during the critical early healing phase has not been fully elucidated.

The inflammatory, proliferative, and early remodeling stages of socket healing occur predominantly within the first three weeks following extraction, making this period particularly relevant for evaluating scaffold–host interactions. Moreover, in vivo validation of a previously optimized 30:70 (w/w) chitosan–gelatin to CHA scaffold formulation is necessary to confirm its biological performance under physiological healing conditions. Therefore, this study aimed to assess the effect of a chitosan–gelatin scaffold reinforced with carbonate hydroxyapatite (30:70 w/w) on extraction socket healing in Wistar rats by analyzing histology and the immunohistochemical expression of bone-associated markers bone morphog protein 2 (BMP-2), Runt-related transcription factor 2 (RUNX2), and receptor activator of nuclear factor κB (RANK) at 7, 14, and 21 days post-extraction. The application of a chitosan–gelatin scaffold reinforced with carbonate hydroxyapatite (CG-CHA) enhances socket healing and leads to higher expression of BMP-2, RUNX2, and RANK compared with sockets without scaffold placement.

2. Materials and methods

2.1. Scaffold preparation

A chitosan–gelatin scaffold reinforced with carbonate hydroxyapatite (CG-CHA) in a 30:70 (w/w) ratio was prepared as previously described.[10,12] Briefly, 0.375 g chitosan, 0.375 g gelatin, and 1.75 g carbonate hydroxyapatite powder were weighed. Gelatin was dissolved in 2 mL of 2% acetic acid at 50 °C under magnetic stirring, followed by gradual addition of chitosan until a uniform gel formed. Separately, the carbonate hydroxyapatite powder was mixed with 0.94 mL distilled water until homogeneous and then incorporated into the chitosan–gelatin solution. The pH of the mixture was adjusted to neutral (pH 7).

The resulting gel was molded into 2 × 5 mm forms corresponding to the rat tooth socket dimensions and frozen at −40 °C for 48 h, followed by freeze-drying at −40 °C for an additional 48 h.[10] Prior to application, scaffolds were sterilized using gamma irradiation.

2.2. Study design and animals

This true experimental study employed a randomized post-test only control group design. Twenty-four healthy male Wistar rats (12–16 weeks old, 200–250 g) were obtained from PT Amaretto Jaya Buana. Animals were acclimatized for one week with standard food and water ad libitum. Rats were randomly assigned into six groups (n = 4 each): Control group receiving extraction only, while Treatment**:** extraction + CG-CHA scaffold. Each group was evaluated at three time points: day 7, day 14, and day 21 post-extraction. All procedures followed appropriate ethical and institutional animal care guidelines.

2.3. Tooth extraction procedure

Animals underwent aseptic preparation using 10% povidone-iodine. General anesthesia was induced intramuscularly with xylazine (0.01 mL/kg BW) and ketamine (0.1 mL/kg BW). The left mandibular incisor was luxated and extracted using rat extraction forceps and a periotome. Sockets were irrigated with sterile distilled water.

In control groups, sockets were sutured without scaffold placement. In treatment groups, a CG-CHA scaffold (30:70 w/w) was inserted into the socket prior to suturing with 5-0 monofilament.[22]

Animals were euthanized at each time point by anesthesia overdose and physical confirmation of death,[23] followed by mandibular resection. Carcasses were buried according to biosafety protocols.[24]

2.4. Immunohistochemistry staining

Specimens were processed for immunohistochemical staining using DAB chromogen (Sigma-Aldrich, USA) at 20 μL/mL concentration. Primary monoclonal antibodies used were BMP-2 (sc-137087), RUNX2 (sc-390351), and RANK (sc-374360) (Santa Cruz Biotechnology™, USA).

Slides were examined under a light microscope (Olympus BX-53, Japan) at 1000 × magnification. Images were obtained using a Sony ZV-E10 digital camera. Positive immunoreactive cells were quantified in 20 random fields per sample.

2.5. Statistical analysis

Data analysis was performed using SPSS version 26.0 (IBM, New York, USA). Expression levels of BMP-2, RUNX2, and RANK between control and CG-CHA-treated groups were compared using independent t-tests. Statistical significance was set at $\mathbf{p < 0.05}$.

3. Results

3.1. BMP-2 expression

BMP-2 expression increased gradually across all time points, with significantly higher levels in the CG-CHA group compared with controls ($p < 0.05$). Staining intensity and positive cell counts were greater in scaffold-treated sockets beginning at day 7 and peaked at day 21, indicating a more active early bone-forming environment (Fig. 1).

Increased BMP-2 expression suggests enhanced early signaling associated with bone formation and reflects a more favorable osteogenic healing environment in scaffold-treated sites.

3.2. RUNX2 expression

RUNX2 expression also increased progressively, with markedly higher levels in the CG-CHA group throughout the study period ($p < 0.05$). Increased RUNX2-positive cells were evident as early as day 7 and continued to rise through day 21, consistent with active osteoblast differentiation and matrix development (Fig. 2).

Increased RUNX2 expression indicates more robust osteoblast presence and maturation in scaffold-treated sockets, supporting improved

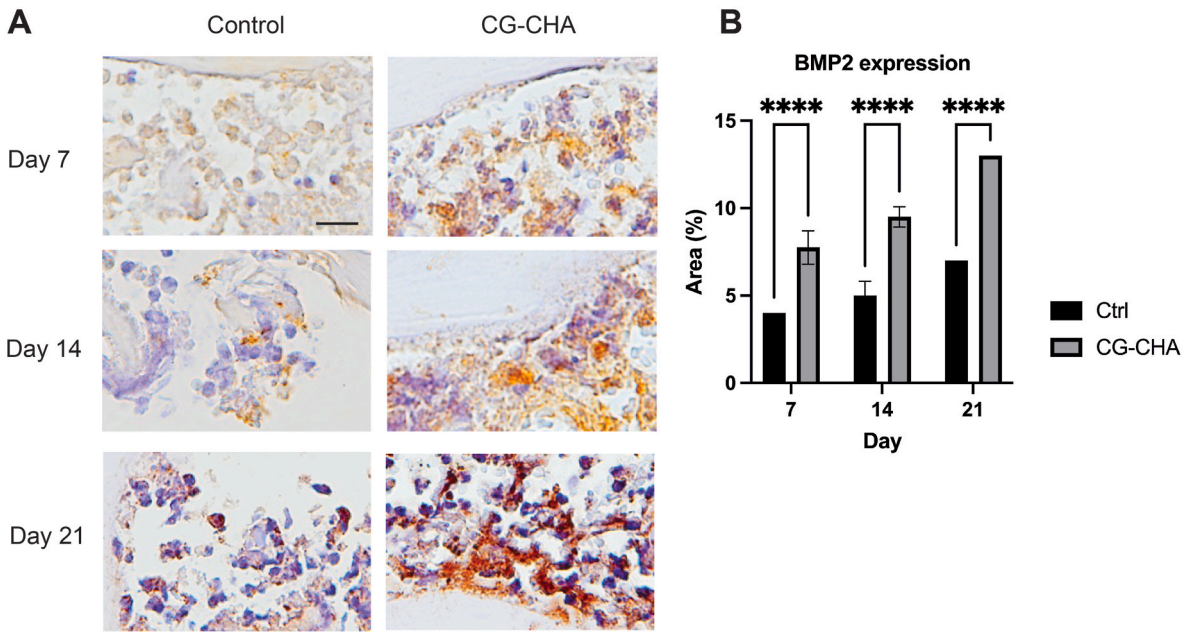

Fig. 1. BMP-2 expression in extraction socket healing. (A) Representative immunohistochemical staining of BMP-2 in extraction sockets at days 7, 14, and 21 in the control and CG-CHA groups. Positive staining is indicated by brown coloration in osteogenic cells and newly forming bone tissue. The CG-CHA group demonstrates visibly stronger BMP-2 staining and more advanced tissue organization across all time points. **(B)** Quantification of BMP-2-positive cells showing significantly higher expression in the CG-CHA group compared with control at days 7, 14, and 21 ($p < 0.05$). Data presented as mean ± SD. Scale bar 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

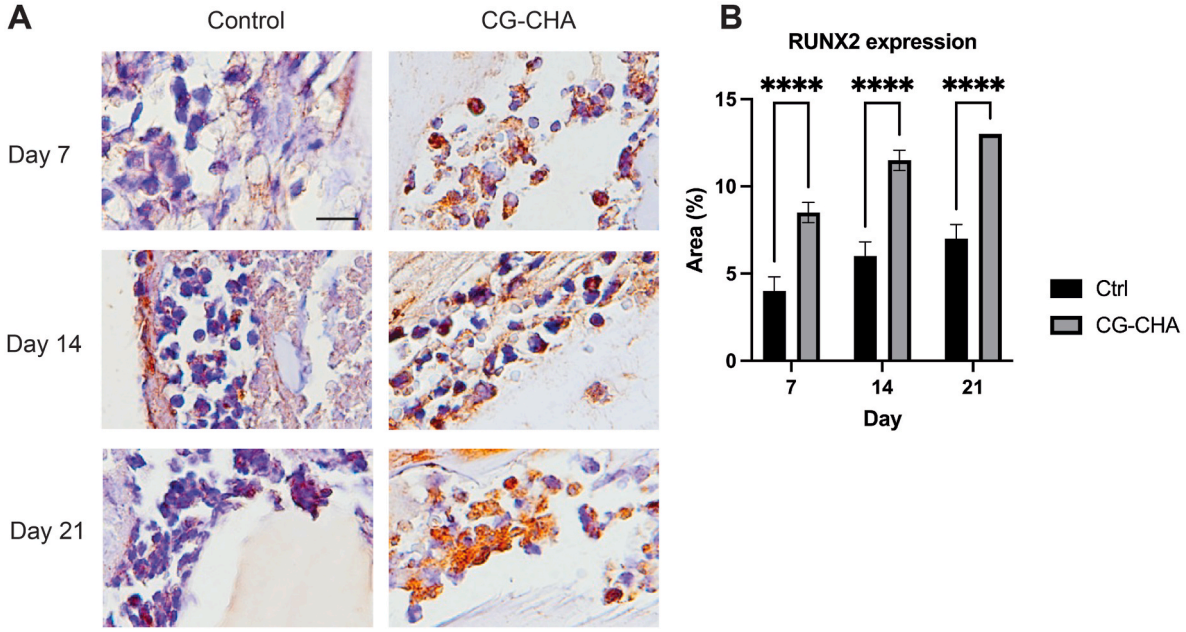

Fig. 2. RUNX2 expression in extraction socket healing. (A) Representative immunohistochemical images for RUNX2 expression at days 7, 14, and 21 in the control and CG-CHA groups. Increased RUNX2-positive cell density and more prominent staining are observed in the CG-CHA group, indicating more active osteogenic cell presence during socket healing. **(B)** Quantitative analysis of RUNX2-positive cells. The CG-CHA group shows significantly higher expression levels than the control group at all time points ($p < 0.05$). Values shown as mean ± SD. Scale bar 100 μm.

intramembranous bone formation.

3.3. RANK expression

RANK expression showed a time-dependent increase, with significantly greater immunoreactivity in the CG-CHA group at all intervals ($p < 0.05$). Staining density increased from day 7 to day 21, with scaffold-treated sockets showing more pronounced expression, consistent with active bone turnover and maturation (Fig. 3).

Increased RANK expression suggests a more dynamic bone remodeling process in scaffold-treated sockets, reflecting ongoing maturation and balanced bone turnover during healing.

4. Discussion

Successful extraction socket healing requires tightly coordinated

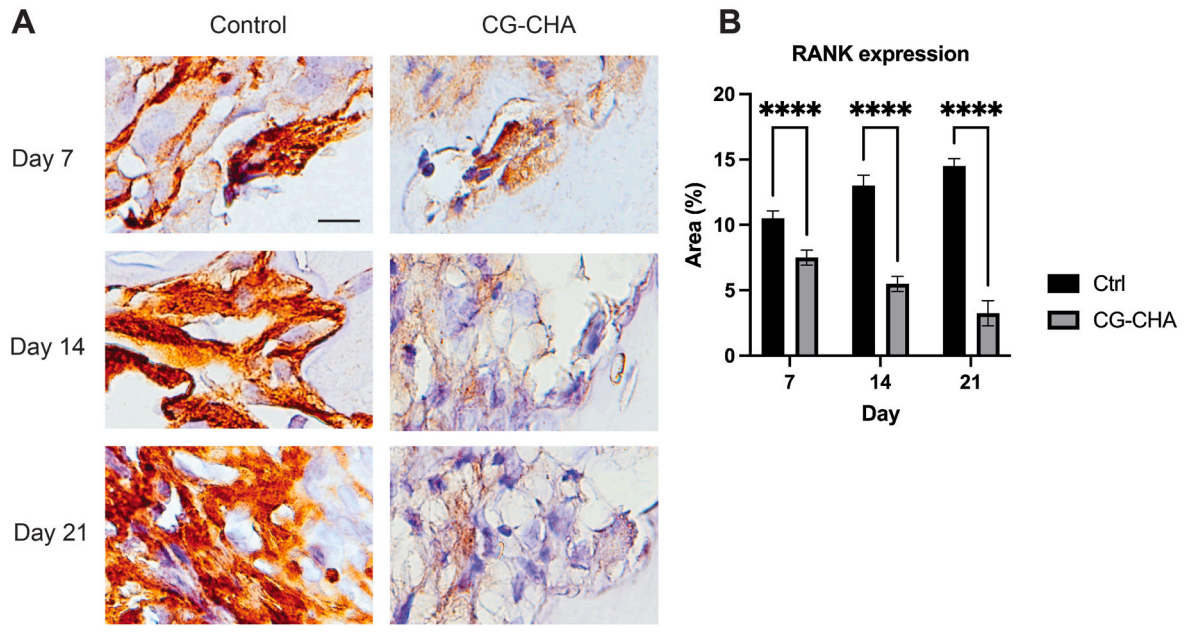

Fig. 3. RANK expression in extraction sockets. (A) Immunohistochemical staining images showing RANK expression at days 7, 14, and 21 in control and CG-CHA-treated sockets. RANK-positive cells are more abundant in the CG-CHA group, particularly at later stages, consistent with increased bone tissue activity during healing. **(B)** Quantification of RANK-positive cells demonstrating significantly higher expression in CG-CHA-treated sockets at all evaluated periods compared with control ($p < 0.05$). Data expressed as mean ± SD. Scale bar 100 μm.

osteogenic differentiation and bone remodeling processes during the early post-extraction period.[25] In the present study, sockets treated with a chitosan–gelatin scaffold reinforced with carbonate-substituted hydroxyapatite (CG-CHA) demonstrated a more favorable biological environment than untreated sockets, as reflected by histological features and increased expression of osteogenesis- and remodeling-associated markers during the first three weeks of healing.

Composite scaffolds that integrate organic polymers with inorganic calcium phosphate phases are designed to recapitulate key aspects of native bone extracellular matrix.[26,27] Gelatin provides cell-adhesive motifs and biocompatibility, chitosan contributes structural integrity and wound-healing support, and carbonate-substituted hydroxyapatite closely resembles biological apatite, facilitating cellular attachment and remodeling. Consistent with previous in vitro optimization of a 30:70 polymer-to-ceramic formulation,[10] the present findings demonstrate that this scaffold configuration also supports early biological responses in vivo within an extraction socket environment.[28]

While prior studies have reported the osteoconductive potential of chitosan- and hydroxyapatite-based scaffolds, the present study extends existing knowledge by focusing on early-stage biological responses during socket healing. The simultaneous evaluation of BMP-2, RUNX2, and RANK expression provides insight into the coordinated activation of osteogenic differentiation and bone remodeling pathways during the critical inflammatory and proliferative phases. This temporal perspective is particularly relevant, as early molecular signaling events play a decisive role in determining subsequent bone regeneration quality.

The observed increases in osteogenic marker expression are consistent with previous reports demonstrating enhanced bone repair in preclinical models treated with chitosan–hydroxyapatite composites and carbonate-substituted hydroxyapatite.[29] However, the present study specifically confirms that a previously optimized CG-CHA scaffold formulation promotes these biological processes within the unique microenvironment of an extraction socket, supporting its translational relevance for alveolar ridge preservation.

From a clinical perspective, alveolar ridge preservation remains essential for maintaining bone volume and optimizing implant and prosthetic outcomes. While xenografts and alloplastic materials remain widely used, naturally derived composite scaffolds such as CG-CHA may offer advantages in terms of biocompatibility, biodegradability, and accessibility. The present findings support the potential of CG-CHA as a biologically active scaffold that enhances early healing events critical for successful socket preservation.

This study focused on early healing phases and relied primarily on immunohistochemical analyses. Radiographic assessment of bone volume and density was not performed. Future investigations incorporating micro-computed tomography, biomechanical testing, and longer observation periods will be necessary to evaluate scaffold stability, degradation behavior, and functional bone regeneration. Additional studies examining vascularization and inflammatory modulation may further clarify the mechanisms underlying CG-CHA–mediated socket healing.

5. Conclusions

The chitosan–gelatin scaffold reinforced with carbonate hydroxyapatite (CG-CHA) supported more favorable socket healing following tooth extraction in Wistar rats, as evidenced by higher expression of osteogenesis-associated markers. The scaffold demonstrated biocompatibility and promoted tissue maturation throughout the early healing period. These findings suggest that CG-CHA has potential as a biomaterial for alveolar ridge preservation and may serve as an effective option to support bone regeneration in clinical dental applications. Further studies with long-term observation, micro-CT evaluation, and functional assessment are recommended to validate these results and clarify the scaffold's translational potential.

Patient's/Guardian's consent

This study did not involve human participants, and therefore, informed consent were not required.

Ethical clearance

This study was approved by the Health Research Ethics Committee, Faculty of Dental Medicine, Universitas Airlangga, Indonesia (Approval No. 1178/HRECC.FODM/X/2023.). All procedures followed

institutional guidelines for the care and use of laboratory animals and complied with the National Guidelines for the Care and Use of Laboratory Animals in Indonesia and the internationally accepted principles for laboratory animal use and care.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the author(s) used ChatGPT in order to check the language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Devi Rianti reports financial support was provided by Airlangga University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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