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RESEARCH ARTICLE

Chicken bone hydroxyapatite enhances collagen density and osteoblast cell number during bone formation of post-extraction socket wound healing process (an in vivo study)

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ABSTRACT

Tooth extraction is a common dental procedure. Osteoblasts (bone-forming cells) and collagen are key indicators of wound healing following tooth extraction. Hydroxyapatite is a calcium-rich material that promotes the secretion of Fibroblast Growth Factor (FGF), Platelet-Derived Growth Factor (PDGF), and Transforming Growth Factor Beta (TGF-β)—all of which play critical roles in the wound healing process. Chicken bones, a natural source of hydroxyapatite, contain approximately 85% calcium phosphate minerals. This study aimed to determine the effect of chicken bone-derived hydroxyapatite on osteoblast cell count and collagen density in post-tooth extraction wounds in Wistar rats. Thirty male Wistar rats were randomly divided into treatment and control groups. Tooth extraction was performed on the lower left incisor of each rat. Hydroxyapatite was prepared by calcining chicken bones at 700 °C to remove organic material. The resulting hydroxyapatite powder was implanted into the tooth sockets of rats in the treatment group, while the control group received no implantation. Both groups were sutured and treated with povidone-iodine. Three rats from each group were sacrificed on days 3, 5, 7, 10, 14, and 21. Histological samples were prepared using hematoxylin-eosin and Mallory's Trichrome staining. Osteoblast cells (100× magnification) and collagen density (400× magnification) were examined using a light microscope and Optilab Viewer, across five fields of view per sample. Two-way ANOVA showed significant differences in both osteoblast cell counts and collagen density between groups and across observation days ($p < 0.05$). Least Significant Difference (LSD) post hoc analysis also revealed significant differences between groups on all observation days ($p < 0.05$). In conclusion, chicken bone-derived hydroxyapatite significantly increases osteoblast numbers and collagen density during the post-extraction wound healing process in Wistar rats.

Keywords: chicken bone hydroxyapatite; collagen; osteoblast; tooth extraction; wound healing

INTRODUCTION

Tooth extraction is the process of removing a tooth from its socket, which can cause injury or trauma.[1] The resulting tissue damage and discontinuity trigger a complex wound healing process that involves several sequential phases: the inflammatory phase, the proliferation phase, and the remodeling or maturation phase.[2]

The inflammatory phase begins with vasoconstriction as part of the hemostatic response. Neutrophils and macrophages migrate to the wound site to phagocytose microorganisms and cellular debris, while osteoclasts initiate bone resorption.[3] Macrophages then produce growth factors such as Platelet-Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF), and Transforming Growth Factor beta (TGF-β).[4] These growth factors stimulate the migration and proliferation of fibroblasts and promote the synthesis of the extracellular matrix, especially type III collagen. They also induce the differentiation and proliferation of mesenchymal stem cells into osteoblasts, which produce type I collagen to form the bone matrix during the proliferation phase.[5,6,7]

In the final remodeling phase, type III collagen is gradually replaced by type I collagen, which has higher tensile strength. This stage also

involves the maturation of bone tissue, driven by a balance between osteoclastic bone resorption and osteoblastic bone formation.[5,8] Thus, in addition to new bone formation through matrix deposition by osteoblasts, the synthesis and replacement of type III collagen are critical for accelerating the healing process, as it is ultimately mineralized and incorporated into newly formed bone.

Although bone tissue can regenerate on its own, bone defects may still develop, necessitating clinical intervention for optimal recovery.[9] Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] is widely used to accelerate bone regeneration due to its excellent biocompatibility and osteoconductive properties.[10] In wound healing, hydroxyapatite can stimulate macrophages to secrete growth factors such as FGF, VEGF, and TGF-β, which promote fibroblast activity, collagen deposition, and osteoblast differentiation and proliferation, thereby supporting accelerated bone formation.[11] Hydroxyapatite is typically applied in the form of scaffold materials with a porous structure that facilitates the growth of new bone tissue and the establishment of mechanically stable interfaces. These pores support cell attachment and proliferation at the injury site, enabling stable bone regeneration.[12,13,14]

Hydroxyapatite can be synthesized from various natural sources, including chicken bones.[15] Chicken consumption in Indonesia is notably high, particularly for broiler chickens.[16] This high consumption rate results in substantial quantities of chicken bone waste, making chicken bones a potentially abundant and sustainable source of hydroxyapatite.[17] Chicken bones are rich in calcium, primarily in the forms of calcium phosphate and calcium carbonate, which make up the mineral composition of hydroxyapatite. Their inorganic content is estimated to consist of approximately 85% calcium phosphate minerals.[18,19] This study aimed to evaluate the effect of chicken bone-derived hydroxyapatite on osteoblast count and collagen density during the alveolar bone wound healing process after tooth extraction in rats. Extracting hydroxyapatite from broiler chicken bone waste is expected to provide valuable insights in the field of dentistry and contribute to reducing agricultural waste.

MATERIALS AND METHODS

This in vivo experimental study received ethical approval (No. 145/UN1/KEP/FKG-RSGM/EC/2023) from the Research Ethics Commission, Faculty of Dentistry, Universitas Gadjah Mada. Thirty-six healthy male Wistar rats, aged 2–3 months, were used and randomly divided into treatment and control groups (n = 18 each). Each group was further divided according to post-extraction observation periods: days 3, 5, 7, 10, 14, and 21, with 3 rats per subgroup.

Broiler chickens used in the study were identified as Gallus gallus by the Systematic Animal Laboratory, Faculty of Biology, UGM. Chicken bones (femur and tibia) were collected from the same farm used for species identification. Three kilograms of chicken bones were cleaned, dried, and crushed into small chips. These were calcined at 700 °C for 4 hours, ground into fine powder, and sieved to obtain hydroxyapatite particles sized at 100 mesh using a ball mill machine. The high-temperature calcination method produced hydroxyapatite with high purity, and X-ray Diffraction (XRD) analysis confirmed a content of 86.9% hydroxyapatite. The powder was then sterilized using ethylene oxide gas.

Wistar rats were anesthetized by intramuscular injection of xylazine (5 mg/kg BW) and ketamine (25 mg/kg BW). The left mandibular incisor of each rat was extracted using an excavator, hemostat, and forceps. For the treatment group, chicken bone hydroxyapatite powder was implanted into the socket until completely filled. The control group did not receive any implants. All extraction sockets were sutured using the simple interrupted technique with 5/0 surgical sutures and treated with povidone-iodine antiseptic. Rats were euthanized on days 3, 5, 7, 10, 14, and 21 post-extraction to collect mandibular samples. The mandibles were processed into histological sections and stained using hematoxylin-eosin for general tissue observation.

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Collagen density and osteoblast cell counts were analyzed using a light microscope with an Optilab Viewer, examining five fields of view per slide. Collagen fibers, appearing as randomly arranged blue fibers, were observed at 400× magnification on days 3, 5, 7, 10, and 14 post-extraction. Osteoblast cells, identified as cuboidal or short cylindrical cells with blue-grey nuclei and basophilic cytoplasm, were observed at 100× magnification on days 5, 7, 10, 14, and 21. Image analysis for collagen density was performed using the ImageJ application. Images of stained slides were converted to grayscale, thresholded to isolate collagen fibers, and analyzed using the "Analyze Particles" function to calculate collagen fiber area. Results were expressed as percentage collagen area relative to total tissue area.

The research data consisted of quantitative variables, namely the number of osteoblast cells and the percentage of collagen fiber density. The data were confirmed to be normally distributed and homogeneous. A two-way Analysis of Variance (ANOVA) was conducted to determine the significance of the effects of broiler chicken bone-derived hydroxyapatite grafts on osteoblast cell numbers and collagen fiber density in the extraction sockets of Wistar rats across different groups. Subsequently, the Least Significant Difference (LSD) post hoc test was used to assess significant differences among the hydroxyapatite application groups. Statistical analysis was conducted using SPSS software at a 95% confidence level (α = 0.05).

Table 1. Mean and standard deviation of collagen density in each group at 3, 5, 7, 10, and 14 days post-tooth extraction in Wistar rats

Day	Mean ± Standard deviation collagen density	
	Treatment group	Control group
3	21.68 ± 0.21	20.32 ± 0.53
5	25.77 ± 0.17	23.77 ± 0.05
7	29.24 ± 0.22	26.40 ± 0.38
10	33.38 ± 0.25	29.24 ± 0.27
14	37.51 ± 0.23	34.45 ± 0.44

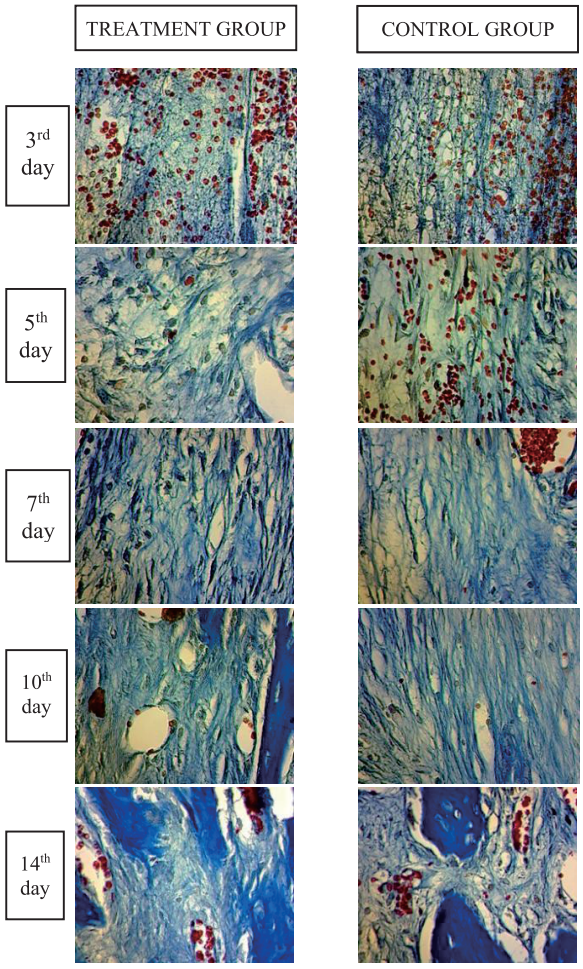

Figure 1. Histological images of collagen fibers (400× magnification) show that the treatment groups exhibited denser collagen compared to the control groups

Table 2. Two-way ANOVA results

Source	Sig.
Group	0.000*
Observation day	0.000*
Group* observation day	0.000*

R Squared = 0.998
* = p < 0.05

RESULTS

In this study, collagen fiber density was first observed on the third day after tooth extraction. The density continued to increase until day 14 post-extraction. The treatment group consistently demonstrated a higher percentage of collagen fiber density compared to the control group (Table 1).

Table 3. LSD Analysis Results ($p < 0.05$)

	C3	T5	C5	T7	C7	T10	C10	T14	C14
T3	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
C3		0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
T5			0.000*	0.000*	0.021	0.000*	0.000*	0.000*	0.000*
C5				0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
T7					0.000*	0.000*	0.989	0.000*	0.000*
C7						0.000*	0.000*	0.000*	0.000*
T10							0.000*	0.000*	0.000*
C10								0.000*	0.000*
T14									0.000*

*significant ($p < 0.05$)
3: 3 days post-extraction; 5: 5 days post-extraction; 7: 7 days post-extraction; 10: 10 days post-extraction; 14: 14 days post-extraction; C: control group; T: treatment group

The results of the two-way ANOVA indicated significant differences in terms of group, observation time, and interaction between group and time with respect to collagen fiber density ($p < 0.05$). This suggests that administration of chicken bone-derived hydroxyapatite had a significant effect on collagen fiber density during the healing period.

The LSD post hoc test (Table 3) revealed that collagen density in the treatment group was significantly higher ($p < 0.05$) than in the control group on days 3, 5, 7, 10, and 14 post-extraction. These findings demonstrate that hydroxyapatite administration effectively enhances collagen fiber density during post-extraction wound healing. Additionally, the LSD test indicated a progressive increase in collagen fiber density from day 3 to day 14, highlighting the role of healing time in collagen fiber formation.

The study also showed that in both the treatment group (administered chicken bone hydroxyapatite) and the control group (treated only with povidone-iodine), osteoblast cell numbers increased from day 5 to day 10, followed by a decrease from day 14 to day 21 after tooth extraction (Table 4). The treatment group showed consistently higher osteoblast cell counts compared to the control group on days 5, 7, 10, 14, and 21 post-extraction.

Table 4. Mean and standard deviation of osteoblast cell counts by group at 5, 7, 10, 14, and 21 days post-tooth extraction in Wistar rats.

Day	Mean ± Standard deviation osteoblast cells	
	Treatment group	Control group
5	42.67 ± 2.00	30.27 ± 3.98
7	55.27 ± 1.85	44.2 ± 3.27
10	69.6 ± 1.51	51.3 ± 4.19
14	67.73 ± 2.25	46.33 ± 2.63
21	60.43 ± 0.72	41.63 ± 1.79

Table 5. Two way ANOVA test results

Source	Sig.
Group	0.000*
Observation day	0.000*
Group* observation day	0.012*

R Squared = 0.968
* = $p < 0.05$

The results of the two-way ANOVA test indicated a significant difference in osteoblast cell counts across all groups and observation days ($p < 0.05$). This finding suggests that the application of chicken bone-derived hydroxyapatite powder significantly influences osteoblast cell proliferation. In addition, the duration of healing also had a statistically significant effect on osteoblast cell numbers.

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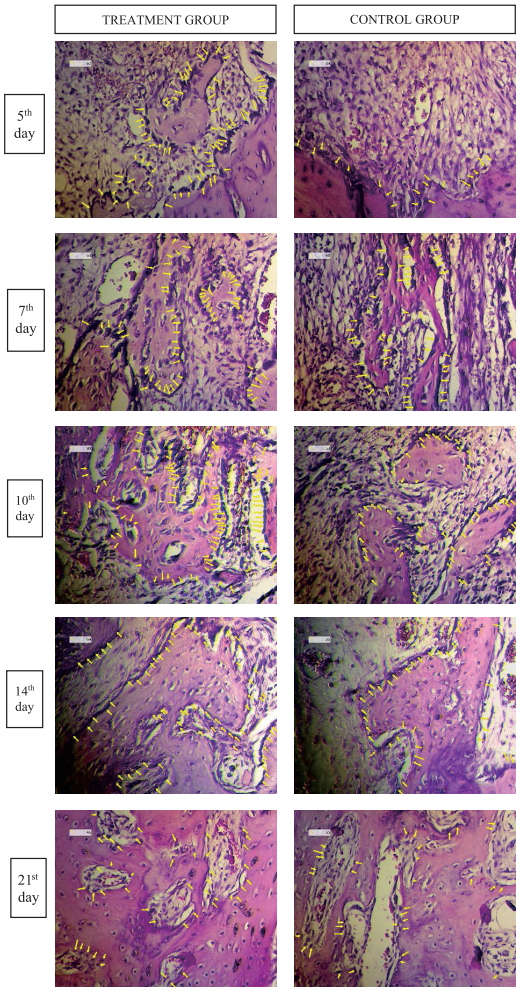

Figure 2. Histological images showing osteoblast cells (indicated by yellow arrows, 100× magnification) demonstrate that the treatment groups had greater osteoblast cell counts than the control groups.

The LSD post hoc analysis (Table 4) revealed significant differences ($p < 0.05$) in osteoblast cell counts between the treatment and control groups on days 5, 7, 10, 14, and 21 post-extraction. This indicates that chicken bone hydroxyapatite effectively increases osteoblast cell numbers during the post-extraction wound healing process. Significant differences in osteoblast cell counts were also observed between the following time points: day 5 and day 7, day 7 and day 10, day 10 and day 14, and day 14 and day 21, in both the treatment and control groups ($p < 0.05$).

DISCUSSION

The results of this study demonstrate that implantation of chicken bone-derived hydroxyapatite enhances both collagen fiber density and osteoblast cell count during the healing process following tooth extraction. Collagen fibers and osteoblasts serve as crucial markers of soft and hard tissue regeneration, respectively, indicating that hydroxyapatite plays a role in stimulating fibroblast proliferation for collagen production and osteoblast differentiation and proliferation for bone formation.

The chicken bone powder used in this study had a hydroxyapatite content of 86.9%. Hydroxyapatite has been shown to stimulate macrophage activation, leading to the release of growth factors such as FGF and TGF-β.[11,20,21]

Table 6. LSD Analysis Results ($p < 0.05$)

	C5	T7	C7	T10	C10	T14	C14	T21	C21
T5	0.000*	0.000*	0.485*	0.000*	0.000*	0.000*	0.000*	0.000*	0.637
C5		0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
T7			0.000*	0.000*	0.000*	0.000*	0.000*	0.026*	0.000*
C7				0.000*	0.004*	0.000*	0.334	0.000*	0.247
T10					0.000*	0.396	0.000*	0.000*	0.000*
C10						0.000*	0.032*	0.000*	0.000*
T14							0.000*	0.003*	0.000*
C14								0.000*	0.041*
T21									0.000*

*significant ($p < 0.05$)
3: 3 days post-extraction; 5: 5 days post-extraction; 7: 7 days post-extraction; 10: 10 days post-extraction; 14: 14 days post-extraction; C: control group; T: treatment group

These growth factors promote the continued proliferation of fibroblasts and osteoblasts, resulting in increased collagen deposition and accelerated bone formation.[11,22]

Hydroxyapatite gradually degrades and releases calcium ions, which increase extracellular calcium levels. This induces the chemotaxis and migration of osteoblasts, and enhances the expression of Cbfa1 (Core-binding factor alpha 1), a key transcription factor that drives the differentiation of mesenchymal stem cells into osteoblasts.[23,24] In this study, hydroxyapatite bone graft powder with a particle size of 150–180 microns was used. The particle diameter of bone graft materials significantly affects their biocompatibility and osteoconductive properties. Particle sizes ranging from 100 to 500 microns are considered suitable for bone grafting, with 150 microns being ideal. However, smaller particle sizes tend to support better bone regeneration, as their larger surface area provides more binding sites for cell attachment, proliferation, and new tissue formation.[25]

The results of the study indicate that although all groups received povidone-iodine application after socket suturing, the percentage of collagen density and number of osteoblast cells were higher in the treatment group that received additional broiler chicken bone-derived hydroxyapatite grafts compared to the control group. This may be because povidone-iodine acts solely as an antibacterial agent, helping to prevent infection by inhibiting bacterial metabolism and oxidizing bacterial cell membranes, which leads to the deactivation of bacterial proteins and DNA/RNA. While it prevents prolonged inflammation, povidone-iodine does not directly stimulate osteoblast proliferation in the ossification process or collagen synthesis by fibroblasts.[26] Therefore, the observed increase in osteoblast cell count and collagen density is attributed to the additional administration of broiler chicken bone graft hydroxyapatite.

The findings support that chicken bone-derived hydroxyapatite enhances collagen density during wound healing after tooth extraction in Wistar rats. This is supported by the presence of abundant collagen fibers (Figure 1), where the treatment group showed a higher collagen density compared to the control group.

Microscopic observations revealed that collagen fibers became visible as early as the third day post-extraction, along with the presence of numerous erythrocytes. Collagen synthesis and deposition begin around day 3, resulting in thin collagen structures.[27] The presence of erythrocytes is associated with the ongoing inflammatory phase, characterized by infiltration of blood and immune cells.[4] From days 3 to 5 and then to 7, collagen fiber density increased, with more prominent fiber formation noted by day 7.[28]

On days 5 and 7, the wound enters the proliferation phase, during which growth factors such as FGF and TGF-β continuously promote fibroblast migration and proliferation, leading to a collagen-rich extracellular matrix.[22] On day 10 post-tooth extraction, collagen fiber density increases further, and mineralized collagen islands begin to appear in the treatment group but not in the control group. Fibroblast-mediated collagen synthesis continues, leading to denser collagen types I and III. Additionally, collagen remodeling processes are evident.[29,30] By day 14, additional mineralized collagen islands appear. Maturation involves collagen remodeling, where mineral salt crystals accumulate in the extracellular matrix, fuse, and eventually calcify to form bone tissue.[31]

These findings further support that chicken bone hydroxyapatite enhances collagen formation. The hydroxyapatite powder used in this study contained 86.9% hydroxyapatite. Upon degradation, it releases calcium ions, which can stimulate fibroblast proliferation.[32] In addition, hydroxyapatite activates macrophages to produce IL-6 cytokines, which in turn promote the expression of FGF and TGF-β.[11,20,21] These growth factors can influence continuous fibroblast proliferation to deposit collagen.[22] Hydroxyapatite can accelerate the wound healing process post-tooth extraction by indirectly accelerating fibroblast proliferation, thus increasing collagen density.[32,33]

The study also demonstrates that the application of broiler chicken bone hydroxyapatite

increases osteoblast cell numbers during socket healing after extraction. This is evidenced by the greater number of osteoblast cells and larger areas of bone formation in the treatment group. Hydroxyapatite promotes the recruitment and migration of osteogenic cells to the site of bone matrix formation and supports osteoblast differentiation and proliferation.[34]

In the histological preparations, osteoblast cells were already visible in all groups by day 5. By three days post-tooth extraction, macrophages begin to produce TGF-β and PDGF, which play essential roles in differentiating osteochondral progenitor cells into osteoblasts, making osteoblasts histologically visible from day 5 onward.[35,36]

Immature bone formation was observed between days 7 and 10 post-extraction, attributed to the increased secretion of FGF-2, which enhances both the proliferation of osteoblast cells and the continued secretion of osteoid by these cells.[37] Bone-forming area became increasingly extensive, especially in the apical and socket wall regions, with a decrease in the number of osteoblast cells observed from day 14 to day 21 post tooth extraction due to the bone already forming. Hard callus formation begins between days 14 and 21 post-extraction, and bone healing in rats is typically completed within 2 to 3 weeks after extraction.[35,38]

The higher number of osteoblast cells in the treatment group indicates a greater production of bone matrix, resulting in faster and more optimal bone formation following tooth extraction. Hydroxyapatite bone graft material is osteoconductive, providing a scaffold for bone cell proliferation and the formation of new bone tissue.[39] Hydroxyapatite also contains calcium, which plays a critical role in regulating mitosis, chemotaxis, differentiation, and proliferation of osteoblasts.[24] Increased extracellular calcium ions promote osteoblast migration and chemotaxis, and calcium further enhances the activation of Cbfa1, a transcription factor that triggers mesenchymal stem cell differentiation into osteoblasts.[23,24]

Therefore, the implantation of chicken bone-derived hydroxyapatite enhances both collagen density and osteoblast cell numbers, which serve as key markers of bone formation and wound healing. A higher density of collagen and increased osteoblast counts suggest that bone matrix and extracellular matrix deposition occur more rapidly, thereby leading to accelerated bone formation and faster healing of the post-extraction socket.

CONCLUSION

The application of broiler chicken bone-derived hydroxyapatite can significantly increase collagen density and osteoblast cell numbers during socket healing following tooth extraction in Wistar rats.

CONFLICT OF INTEREST

The authors declare no conflict of interest with the data contained in the manuscript.

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