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Increased number of osteoblasts and new bone formation in rat's tooth socket implanted with nanocrystalline hydroxyapatite from pensi shells

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Abstract

Preserving alveolar bone with osteoconductive biomaterials may prevent excessive resorption in the post extraction tooth socket. In this study, nanocrystalline hydroxyapatite (nHA) made from pensi shells *(Corbicula moltkiana)* through sol-gel method was implanted in the post extraction rat's tooth socket. The effect of nHA on the number of osteoblasts and new bone formation in the post extraction sockets were then evaluated. The study involved a total sample of 16 rats in 4 groups: group I (control day 14) and group II (nHA day 14), group III (control day 28) and group IV (nHA day 28). XRD and SEM analysis results confirmed a formation of pure hydroxyapatite materials with crystal and particle size of 41.78 nm and about 100 nm, respectively. The results showed a significant difference in the number of osteoblasts and new bone formation in bone healing after tooth extraction in the control group and the nanocrystalline hydroxyapatite implantation group on day 14 and 28 ($p < 0.05$). Group IV showed the highest number of osteoblasts by 43.6 ± 2.27 and the highest new bone formation $1{,}407.4 \pm 0.57$. Following the analysis results of XRD, SEM, and histological picture of nHA implanted tooth socket, this study suggests that nHA made from pensi shells is potential to be developed as biomaterial implant for post-extraction tooth socket.

Keywords: *Nanocrystalline hydroxyapatite, shells, osteoblasts, new bone formation, tooth socket*

1. Introduction

Tooth extraction is one of the routine procedures performed in dental practice. The procedure is done when teeth can no longer be treated with other treatments. Tooth extraction may result in various complications such as damaged soft and hard tissue structures in the oral cavity and alveolar bone resorption. Alveolar bone resorption could reduce the alveolar bone's dimensions and quality. [1,2] The thin alveolar bone in the labial area causes large dimensional changes, especially in the anterior jaw area while the reduction in the vertical height of the alveolar bone in the posterior jaw area often complicates treatment at the rehabilitation stage, especially for dental implants. Complication occurs because dental implants require proper location and placement in alveolar bone. Resorption of alveolar bone occurs gradually, but the first few month resorption after extraction is very prominent. [2] Tan et al. (2011) reported that in the first 6 months after tooth extraction horizontal and vertical bone resorption were 29 – 36% and 11 – 22%, respectively. [3]

A possible technique to speed up the recovery process and avoid excessive resorption of alveolar bone is preservation of alveolar such as minimizing trauma during extraction, soft and hard tissue grafting, use of membranes, and immediate implant placement. [4,5] Preservation of alveolar bone with bone graft material is popular because it facilitates new bone formation as well as avoiding disproportionate resorption of alveolar bone. The material used remains the hot topic in different studies to find the most effective material. [4,6,7]

The gold standard for bone graft material is autogenic bone graft material which is taken from the same individual. This material is good because the grafted material is living tissue and intact cells. It does not trigger an immunogenic reaction but may increase morbidity because it requires additional surgery for donor organ harvest, in addition to the limited form and number of organs harvested. Allogeneic bone grafts using materials from different individuals and xenogenic bone grafts from different species may generate problems related to the patient's

immune response and spreading of diseases. Synthetic-made alloplastic bone graft materials which are bioactive and biocompatible with the body are currently under development. [5,6,8]

In the alveolar bone's preservation using bone graft, the interaction of biomaterial with cells and tissues after tooth extraction begins immediately after the implantation into the tooth socket. Healing of the post-extraction socket will go through 4 phases: hemostasis and coagulation, inflammation, proliferation, and the modelling and remodelling phase. [9,10]

The above points stimulate the researchers to determine the effects of nanocrystalline hydroxyapatite (nHA) implantation from pensi (*Corbicula moltkiana*) shells on the number of osteoblasts and new bone formation in bone healing after tooth extraction. The nHA used in this study was synthesized from pensi (*Corbicula moltiana*) shells using a new combination of previous works. [11,12]

2. Methods

2.1 Materials

CaO as calcium precursor was extracted and prepared from pensi shells (*Corbicula moltkiana*). Firstly, the pensi shells were washed using tap water, dried, and ground to obtain coarse powder. The powder was calcined for 5 hours at 900 °C to obtain CaO. HNO_3, NH_4OH, and $(NH4)_2HPO_4$ used were analytical grade from Merck.

2.2 Hydroxyapatite synthesis

Hydroxyapatite (HAp) was synthesized based on the procedure reported by Azis et al. and Labanni et al. in the stages as follows: 4.2 grams of CaO was added with 75 mL of 2 M HNO_3, then was stirred 500 rpm for 15 minutes at 85 °C. The solution was then filtered. A total volume of 250 mL of 0.18 M $(NH_4)_2HPO_4$ solution was added dropwise to the filtered solution while stirring at 500 rpm at 110 °C. During the reaction, the pH of the mixture was adjusted to 11 by the addition of NH_4OH. After adding all $(NH_4)_2HPO_4$, the mixture was continuously stirred for

5 hours at 100°C, left for 24 hours, then filtered to form gel. The obtained gel was dried in an oven at 110 °C for 4 hours. The obtained solid was then mashed into powder then calcined at 800 °C for 3 hours to obtain hydroxyapatite powder [12,13]. The characterization was carried out using X-Ray difraction (XRD) (XPERT PRO Pananalytical PW30/40) and Scanning Electron Microscopy (SEM) (HITACHI S-3400N). The schematic ilustration of hydroxyapatite fabrication from pensi shells is served in Figure 1a.

2.3 Preparation of experimental animals

The use of experimental animals has been approved by the Research Ethics Committee of the Faculty of Medicine, Andalas University, Padang, Indonesia as stated in the permit letter No. 668/KEP/FK/2019. 16 male rats weighing +200 g were divided into 4 groups, each of which consists of 4 rats: group I (control day 14), group II (nHA day 14), group III (control day 28), group IV (nHA day 28). Ad libitum were then given to the rats.

2.4 Anesthesia and surgical procedures

General anesthesia was performed with the use of ketamine and xylazine. The tooth extraction procedure refers to the protocol as reported by Rakhmatia et al. [14]. The right lower central incisor was cut every 3 days up to 2 times in a row before tooth extraction was performed. Cutting was performed at the gingival margin using a diamond disk bur to remove the retention of the periodontal ligament and facilitate tooth extraction. In the third period after cutting the teeth, the tooth was carefully extracted using a needle holder with a controlled movement and the right lower central incisor was extracted in a horizontal direction along the tooth axis (Figure 2.a,2.b and 2.c).

In the treatment group, the post-extraction tooth socket was filled with HA using an amalgam gun and compacted using an amalgam condenser while in the control group the post-extraction tooth socket was left empty. Rats were given gentamicin antibiotic for 3 days and analgesic novalgin for one day with a dose of 0.3 ml each after tooth extraction. 4 rats in the

treatment group and the other 4 rats in the control group were euthanized on day 14 while the remaining 4 rats in the treatment group and control group were euthanized on day 28. Euthanized was carried out by administering a lethal dose of anesthetics. The rat's right lower jaw was taken and put in a solution of formalin buffer fixation to make histological preparations.

2.5 Histological analysis

The histological preparations were made through the process of decalcification, dehydration, clearing, paraffin infiltration, embedding and cutting with a thickness of 4 µm in the transverse direction parallel to the sagittal plane using a microtome. The tissue was then stained with hematoxylin-eosin. Observation of the number of osteoblasts and new bone formation was carried out using a light microscope (Olympus Cx33). The number of osteoblasts was observed in the region of interest (ROI) is 3 areas in the same width and in 1mm distance beginning from the base of the tooth socket such as proximal (1), middle (2), and distal (3) from the tooth socket (Figure 2.d).

New bone formation was first measured by capturing the preparation with a Sony Beta Exmor CMOS 3.1mp camera. Then, a measurement of the newly formed woven bone was done by drawing a straight line from the base of the woven bone in the apical area of the base of the post-extraction tooth socket to the farthest extent of the formed new bone. It was perpendicular to the center of the post-extraction tooth socket using the betaview.ink program at 40x magnification. Measurements were made at 8 different points on the micrometer scale.

2.6 Statistical analysis

Statistical analysis was conducted using SPSS 18.0 with a significance level and confidence level of 0.05 ($p = 0.05$) and 95% ($\alpha = 0.05$), respectively. Shapiro-Wilk test was carried out to test and analyze the normality of the data while Levene test was carried out to analyze the

homogeneity of population variation. Normally distributed and homogeneous data will be tested by One-way Anova to determine the difference between the independent and dependent variables. In case that a significant difference is found, Least Significant Difference test will be carried out.

3. Results

3.1 Hydroxyapatite characterization

XRD analysis was carried out to study the purity of as-synthesized hydroxyapatite through phase dan crystallinity (Fig. 1.b). Some dominant peaks are observed in 2θ of 25.8, 31.8, 32.2, 32.9, 34.1, 39.8, 46.7, and 49.4°. These peaks are in accordance with with ICSD standard No. 26204, referring to hexagonal crystal structure of hydroxyapatite. The crystallite size of hydroxyapatite was calculated using Debye-Scherrer equation and found to be 41.78 nm.

Fig 1.c show the micrograph of the sample. It was observed that the sample contains evenly distributed particles which have sphere-like shape and mean diameter of about 100 nm.

3.2 Number of Osteoblasts

The results of the Shapiro Wilk test on the number of osteoblasts showed a normal data distribution ($p>0.05$). The homogeneity test showed a significant variation result of $p = 0.071 > 0.05$. One-way Anova parametric test resulted in the value of $p = 0.000 < 0.05$. The average number of osteoblasts on day 14 and 28 is served in Table 1. The LSD test showed that there was a different effect on the measurement of the number of osteoblasts in all groups with $p < 0.05$. The highest number of osteoblasts was obtained by Group IV with a value of 43.6 ± 2.27. Figure 3 showed a histological picture of the tooth socket after rat tooth extraction on day 14, while Figure 4 showed the histology view of post-extraction socket with hematoxylin-eosin staining in group I, II, III and IV.

3.3 New Bone Formation

The results of the Shapiro-Wilk normality test on new bone formation showed a normal data distribution with p>0.05. The homogeneity test of variation also revealed a significant result with p = 0.100>0.05. These results suggested that the data taken from all control and treatment groups on day 14 and 28 were homogeneous. One-way Anova parametric test shows the p value of 0.001 < 0.05. The LSD test showed that there was a different effect on the measurement of new bone formation in all groups with p <0.05, except for the control group on day 14 and control group on day 28. Group IV showed the highest new bone formation 1407,4 $\pm$ 0.57. Figure 5 showed the new bone formation in woven bone after tooth extraction in all groups.

4. Discussion

The diffraction pattern of synthesized hydroxyapatite in the XRD analysis result was identical with the diffraction pattern of commercial hydroxyapatite that was previously reported by Roman-Lopez et al. [15] The result clearly suggested that high crystallinity HAp without any impurities has been successfully synthesized from pensi shells as calcium precursor. The crystallite size of the as-synthesized sample (41.78 nm) confirmed a formation of nanocrystalline hydroxyapatite.

Furtherly, SEM analysis was carried out to study the surface morphology of the sample. The result showed a formation of sphere-like particles with a mean diameter of about 100 nm. Those nanoparticles have smaller size than HAp nanoparticles from fish bones and bovine femur which were previously reported. [16,17] The small size of particles is expected to significantly affect the properties and performance of materials including number of osteoblasts and new bone formation.

The homogeneity test of the number of osteoblasts showed a p value of 0.071>0.05, suggesting that data of all control and treatment groups on day 14 and 28 were homogeneous. In addition, One-way Anova parametric with p value of 0.000<0.05 suggested that implantation of nHA affected the formation of the number of osteoblasts in bone healing after tooth

extraction. The osteoblasts are known to participate actively in biomaterial mineralization by producing calcium phosphate containing vesicles. Osteoblasts synthesize and secrete subsequently type I collagen, alkaline phosphatase, and other non-collagenous extracellular bone matrix proteins such as osteocalcin, osteopontin, osteonectin, and bone sialoprotein through osteogenic differentiation phase. [18] The increased number of osteoblasts was induced by the implantation of pensi shells-based hydroxyapatite since it has nanocrystalline structure (with size of ≤ 100 nm), good biocompatibility, and chemical bonding with bone tissue, biointegrative and osteoconductive. [19–21] The Hydroxyapatite nanocrystals with average size of about 100 nm have close properties with natural hydroxyapatite in bone. It provides closer and more contacts with the surrounding tissue, absorbable properties, and high total of molecules on the surface. [19,22]

Nanocrystalline hydroxyapatite can stimulate osteoblast activities as well as growth of new bone in tooth socket healing. The results of this study is in accordance with those of Cestari et al. who synthesized nHA from shells and eggshells which showed a good cell adhesion and did not provide cytotoxicity. [23]

In the study of the new bone formation, One-way Anova parametric test shows the p value of $0.001 < 0.05$. It means that there was an effect of implantation of nHA on the formation of new bone in post-extraction bone healing of white wistar rats on day 14 and 28 (Table 2). The bone healing process in the implantation of nHA proceeds in the stages of socket healing. The implantation of the biomaterial leads to the cellular interactions. There are 4 phases in the recovery process following tooth extraction. The first is hemostasis and coagulation phase, which occurs immediately after the tooth is extracted. Microvascular damage and bleeding process enables blood to fill the post extraction tooth socket. Blood clots and platelets play an important role in this phase as they contain sufficient number of cytokines, chemokines,

interleukin families and growth factors. In this phase the bone graft will be trapped in the fibrin mesh. [9]

The second phase is the inflammatory phase. This phase starts from the beginning of the wound and lasts until about the fifth day of the healing process. In this phase, leukocyte cells carry out debridement of bacteria and necrotic tissues. The presence of bone grafts in this phase triggers the migration of inflammatory cells and osteoclasts on their surface and stimulates a slight resorption. [9] The next phase is the proliferative phase, in which tooth socket is filled with granulation tissue such as macrophages, matrix fibroblasts, and new blood vessels, as well as osteoblasts and mature collagen. Day 14 days after tooth extraction, in addition to mineralized bone, new woven bone formation is spotted where provisional matrix is found in the tooth socket. In the present study, Nanocrystalline HAp showed the ability to increase osteogenesis by facilitating osteoblast cells and new blood vessels to develop to form new bone on day14 (Figure 3).

New woven bone with primary osteons will fill the tooth sockets 30 days after the extraction. The new bone interacts with the existing on the socket wall, where the bone graft stimulate the new bone to grow. [9,10,24] The last phase is modelling and remodelling. The first refers to changes in bone structure and tissue which affects architectural and shape modifications. On the other hand, remodelling takes place without the above modifications. Intra-membranous ossification of the newly formed tissue characterizes the remodelling phase in which the socket is filled with newly generated bone. Those two processes are the output of the interactions between osteoclasts and osteoblasts. The interactions involve modulations of macrophage colony stimulating factor (M-CSF), receptor activator of nuclear factor kappa B (RANK), receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotogerin (OPG). [9]

The results of this study correspond to Alhussary et al. [25] that nHA may increase new bone formation without causing excessive inflammatory responses. This study managed to demonstrate the effects of pensi shells-originating hydroxyapatite on the number of osteoblasts and new bone formation after tooth extraction despite all limitations. This hydroxyapatite is worth using as bone graft material for the preservation of alveolar bone. Further study is necessary to see the degree of bone maturation and material resorption in a longer time span, and to see various proteins are involved as biological responses to this material.

5. Conclusion

Following the characterization result and analysis, it is concluded that nanocrystalline hydroxyapatite implantation originating from pensi *(Corbicula moltkiana)* shells strongly affects the number of osteoblasts and new bone formation in bone healing following tooth extraction of rats on day 14 and 28. Nanocrystalline hydroxyapatite implantation group showed the highest increase in the number of osteoblasts and new bone formation. The nHA is worth using as bone graft material for the alveolar bone's preservation.

Declaration of Interest

The author report no conflict of interest.

Acknowledgement

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Table 1. Average number of osteoblasts

Group	**Average number of osteoblasts** $\mathbf{x \pm SD}$	***p - value***
I	18.3 ± 0.86	0.00*
II	35.5 ± 2.88	
III	21.4 ± 0.57	
IV	43.6 ± 2.27	

x: mean group.

SD: standard deviation.

p: Anova test (α= 95%).

*: significantly different.

Table 2. Average new bone growth

Group	Average New Bone Growth (µm) ± SD	*p-value*
I	201,5 ± 0.05	0.001*
II	805,0 ± 0.18	
III	533,7 ± 0.09	
IV	1407,4 ± 0.7	

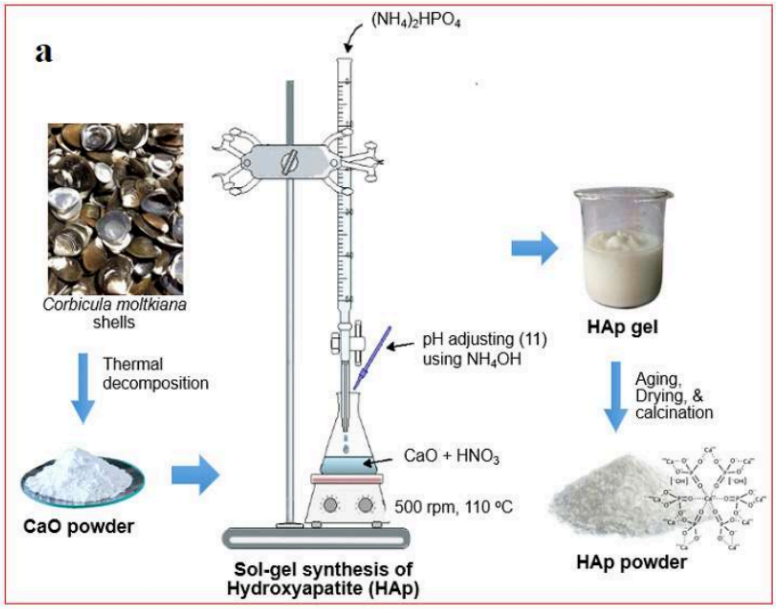
x: mean group.

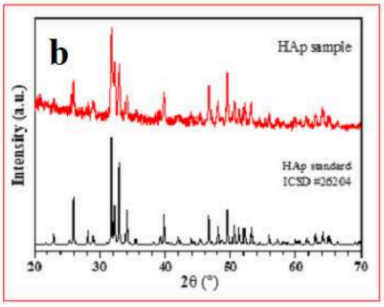
SD: standard deviation.

p: Anova test (α= 95%).

*: significantly different.

10
Figure 1.


a
$(NH_4)_2HPO_4$
Corbicula moltkiana shells
Thermal decomposition
CaO powder
pH adjusting (11) using NH_4OH
$CaO + HNO_3$
500 rpm, 110 °C
Sol-gel synthesis of Hydroxyapatite (HAp)
HAp gel
Aging, Drying, & calcination
HAp powder


b
HAp sample
HAp standard ICSD #26204
Intensity (a.u.)
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30
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70
2θ (°)

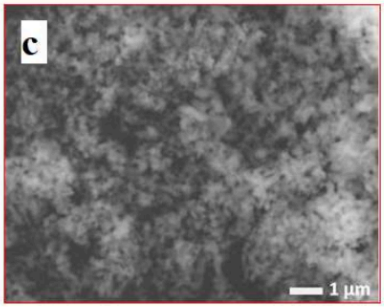
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Figure 2

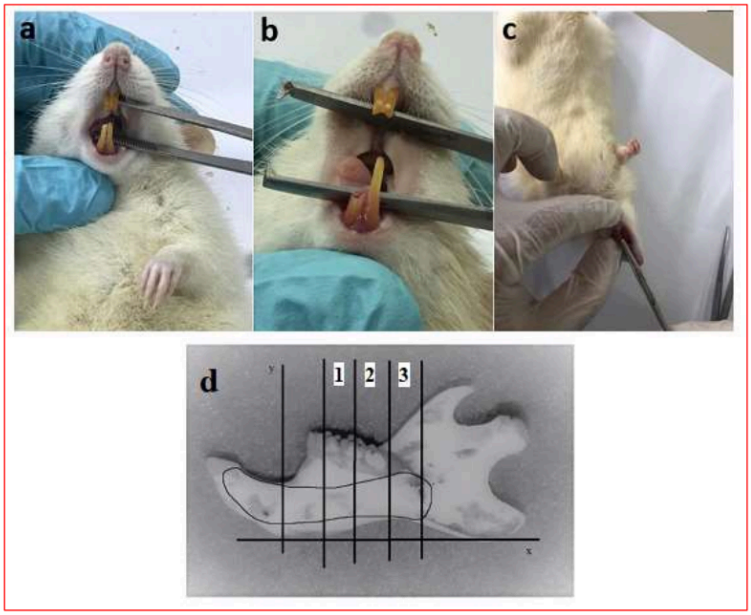

Figure 3

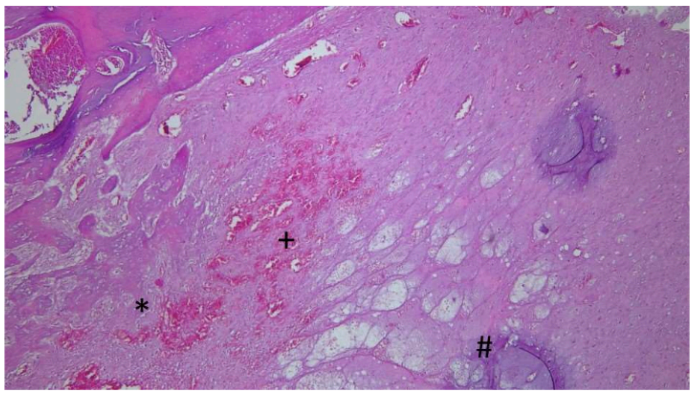

Figure 4

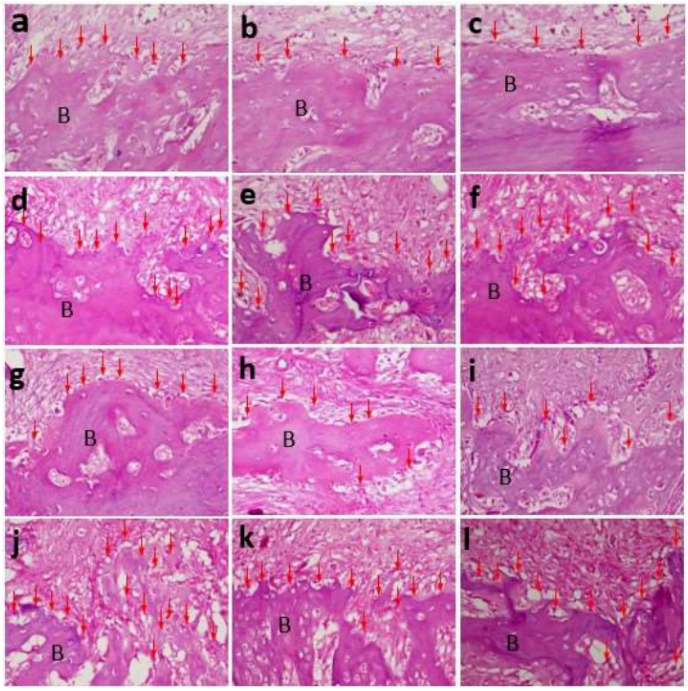

Figure 5

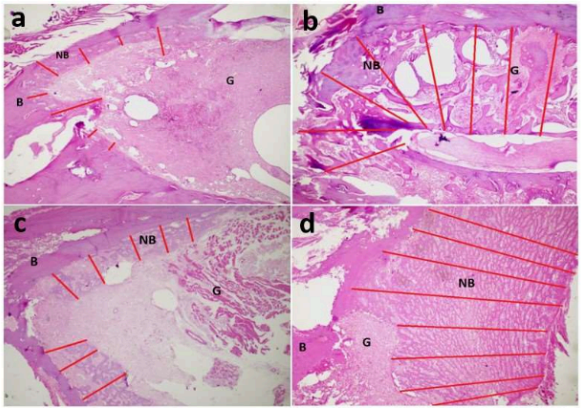

Figure captions:

Figure 1. Schematic illustration of synthesis process of hydroxyapatite, a. synthesis process of hydroxyapatite, b. XRD pattern of as-synthesized hydroxyapatite, c. SEM images of hydroxyapatite sample at 10,000x .

Figure 2. Lower incisor in rat a. before cutting, b. after cutting to the gingival margin, and c. rat tooth extraction, d. ROI of region 1, 2 and 3. The plane (x) is a parallel plane to the mandibular plane and the plane (y) is a perpendicular plane to the mandibular plane

Figure 3. Histological picture of the tooth socket after rat tooth extraction on day 14 (40x magnification). (#) = HAp, (+) = blood vessels, (*) = new bone

Figure 4. Histology of post-extraction socket with hematoxylin-eosin staining at 40x magnification. Osteoblast cells are shown by red arrows. B = Bone matrix. Figure a. b. and c. respectively shows apical, middle and cervical areas in the control group on day 14 (Group I). Figure d, e and f show the apical, middle and cervical areas day 14 treatment group (group II). Figure g, h and i respectively shows apical, middle and cervical areas in the control group on day 28 (group III). Figure j, k. and l show apical, middle and cervical areas in the treatment group on day 28 (group IV)

Figure 5. Measurement of new bone formation in woven bone after tooth extraction. (a) Control group on day 14 (group I), (b) nHA group on day 14 (Group II), (c) Control group on day 28 (group III), (d) nHA group on day 28 (group IV). B = Bone, NB = New bone, and G = Granulation tissue

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