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Original Article

Alveolar bone preservation using a combination of nanocrystalline hydroxyapatite and injectable platelet rich fibrin: A Study in Rats

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"new bone formation of nanocrystalline Hap and IPRF"

Background and purpose: Alveolar bone resorption is a post-extraction complication where there is a reduction in the dimensions and quality of the alveolar bone. One way to accelerate healing and prevent excessive resorption is by preserving alveolar bone using nanocrystalline hydroxyapatite (HA) and injectable platelet-rich fibrin (IPRF). This study aimed to examine the effects of implantation of a combination of HA and IPRF on the expression of Tartrate Resistant Acid Phosphatase (TRAP) as an indicator of resorption, and Alkaline Phosphatase (ALP), Osteocalcin (OCN) and New Bone Formation as an indicator of bone grow

Materials and method: This research is purely experimental with a post-test control group design. 32 male rats had their upper right incisors extracted under general anesthesia (ketamine + xylazine) and then were divided into a control group where the socket was left empty, the HA group where HA was implanted, the IPRF group where IPRF was injected and the HA-IPRF group where the HA-IPRF combination was implanted. Implantation of the material was carried out using a micropipette. Decapitation was done on day 14 and day 28 in each group and the jaws of each rat were subjected to immunohistochemical analysis to see the expression of TRAP, ALP, OCN, and histological analysis using the Image Raster 3 to see the area of woven b formation. Kruskal-Wallis statistical tests were performed for TRAP, ALP, and OCN followed by the Mann-Whitney test, while the Annova test was used for n bone formation and continued with the LSD test.

Results: The results showed a decrease in TRAP expression in the HA-IPRF group compared to the control group although it was not significant on day 14 ($p = 0.074$) but a significant decrease was se on day 28 ($p = 0.017$). The study also showed an increase in ALP and CN in the HA-IPRF group on day 14 and day 28 compared to the co ol group although it was not significant. New bo ormation suggested a significant increase in the HA-IPRF group compared to the control group on day 14 ($p = 0.001$) and day 28 ($p = 0.001$).

Conclusion: HA-IPRF implantation can surpress alveolar bone resorption which is indicated by a decreased TRAP expression, and it can increase bone growth as indicated by increased expression of ALP, OCN, and new bone formation.

Keywords: ***nanocrystalline hydroxyapatite, TRAP, ALP, OCN, new bone, post-extraction healing***

1. Introduction

Morphological changes in alveolar bone have been known and explained by a number of pre-clinical and clinical studies, where preserving alveolar bone is one of the ways to prevent the changes (1–4). Alveolar bone preservation aims to minimize bone loss and changes in alveolar bone dimensions after tooth extraction. The success of this procedure will support the rehabilitation process during the dental implants, which must be placed in the right vertical and horizontal dimensions on the alveolar bone (5,6). Accurate procedure will ensure strong and durable dental implants in the oral cavity. Not only preventing excessive resorption of alveolar bone, alveolar bone preservation using bone graft can also increase new bone growth and accelerate the healing process (6,7).

Various studies have tried to find the ideal bone graft (8). Autograft remains the gold standard because it provides cells and alive tissues and does not cause immunogenic reactions to the host. Harvesting donor organs, on the other hand, requires additional surgery that will increase the risk and morbidity in patients. Alternatively, bone grafts can also be obtained from other individuals or allografts and taken from different species or xenografts. The two bone grafts are advantageous because they can be produced abundantly but have problems in disease

transfer and immunogenic reactions (9). At present, synthetic bone graft or alloplastic bone graft is being developed as the alternative bone grafts (10).

Nanocrystalline hydroxyapatite (HA) is a synthetic material made from natural or artificial sources, which resembles natural hydroxyapatite in bone better than commercial hydroxyapatite. Besides, HA is also known to have a close contact with surrounding tissues, and is bioresorbable, surface free energy and high binding energy (11,12). This material is known to have osteconductive properties which work as a scalfold for new bone growth. However, HA has limited osteoinductive properties, because of which researchers try to combine HA with various materials to improve its osteoinductive ability such as injectable platelet rich fibrin (IPRF). IPRF is a platelet concentrate obtained autologous or blood from its own host centrifuged at low speed. IPRF is rich in cytokines and growth factors that can increase cell proliferation and differentiation, promote angiogenesis, act as a matrix for tissue growth, and regulate inflammatory and anti-infective reactions. Growth factors from IPRF such as Platelet Derived Growth Factor (PDGF), Transforming Growth Factor (TGF-β) and Insulin Growth Factor (IGF) are essential in bone healing and regeneration (8).

Healing in the socket following tooth extraction consists of several phases inxluding the hemostasis and coagulation, the inflammatory, the proliferation, and the modeling and remodeling phases, all of which can overlap (2). Some biomarkers secreted during the bone healing process include tartrate-resistant acid phosphatase (TRAP) which plays a role during bone resorption, alkaline phosphatase (ALP) and other noncollagen extracellular bone matrix proteins such as osteocalcine (OCN) which contributes in osteogenesis (13). Based on the above background, the author is interested in examining the effects of combined nanocrystalline hydroxyapatite and IPRF implantation on the expressions of TRAP, ALP, OCN and new bone formation on post-extraction bone healing in wistar rats.

2. Materials and Methods

2.1 Materials

Hidroxyapatite

CaO was produced and taken from the pensi shells as a calcium precursor *(Corbicula moltkiana)*. The pensi shells were first dried, cleaned with tap water, and then ground into a coarse powder. To produce CaO, the powder was calcined for five hours at 900 °C. HNO_3, NH_4OH, and $(NH4)_2HPO_4$ used were analytical grade from Merck.. In Figure 1, pensi shell images are shown. (a).

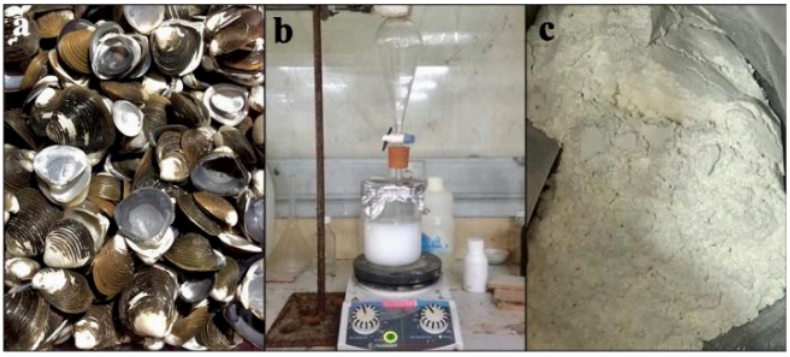


Figure 1. a. Pensi shells, b. hydroxyapatite synthesis, and c. as-synthesized hydroxyapatite powder after calcination

2.2 Hydroxyapatite synthesis

Based on the method described by (14,15) Azis et al. and Labanni et al., hydroxyapatite (HAp) was synthesized in the following steps: 75 mL of 2 M HNO_3 and 4.2 grams of CaO were added, followed by 15 minutes of 500 rpm stirring at 85 °C. The remedy was next filtered. The filtered solution was mixed at 500 rpm at 110 °C with the addition of a total volume of 250 mL of a 0.18 M $(NH4)_2HPO_4$ solution. The mixture's pH was raised to 11 during the reaction by the addition of NH_4OH. All of the $(NH4)_2HPO_4$ was added, and the mixture was then stirred continuously for five hours at 100°C. After being left for 24 hours, the mixture was filtered to form gel. In an oven set to 110 °C for four hours, the gel was dried. To create hydroxyapatite powder, the obtained solid was ground into powder and then heated to 800 °C for three hours (14,15). X-ray difraction (XRD) (XPERT PRO Pananalytical PW30/40) and scanning electron microscopy (SEM) (HITACHI S-3400N) were used to perform the characterization.

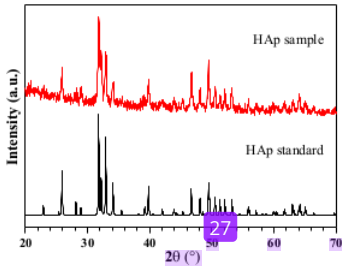


Figure 2. XRD pattern of hydroxyapatite

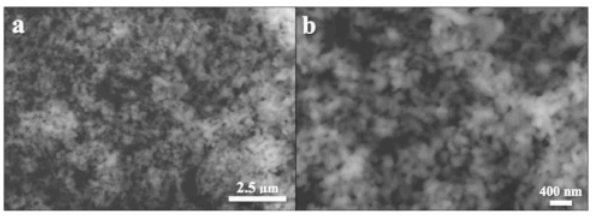


Figure 3. SEM images of hydroxyapatite sample at a. 10,000x, b. 20,000x magnification

Injectable platelet rich fibrin (IPRF)

The rat blood was taken before tooth extraction. Using ketamine and xylazine, general anesthesia was performed and then 1 ml of blood was taken from the periorbital vein. Blood sample of each rat were centrifuged with a centrifugation device for 3 minutes at a speed of 700 rpm, after which IPRF was separated from the blood plasma. The IPRF was made based on the protocol in the study of (16).

Combination of nanocrystalline HA and IPRF

The combination of nanocrystalline HA in the form of 20 mg powder was mixed into 0.2 ml IPRF to form suspension.

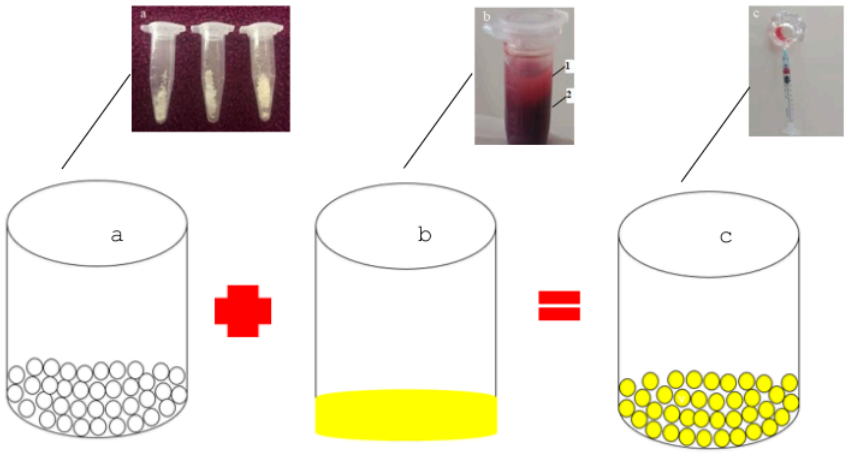


Figure 4. a. Nanocrystalline hydroxyopathy, b. (1) IPRF, (2) Blood, c. Combination of HA and IPRF

2.3 Preparation of experimental animals

Referring to the authorization letter No. 338/UN.16.2/KEP-FK/2021, the use of experimental animals has been allowed by the research ethics committee of the faculty of medicine at Andalas University in Padang, Indonesia. Group I (control day 14), Group II (control day 28), Group III (IPRF day 14), Group IV (IPRF day 28), Group V (HA day 14), Group VI (HA day

28), Group VII (HA-IPRF day 14), and 32 male rats weighing ±200 g were divided into 8 groups, each of which comprises 4 rats. The rats were then given food and water by ad libitum.

2.4 Anesthesia and surgical procedures

Ketamine and xylazine were used to perform general anesthesia. The method used to extract the teeth follows the technique described by Rakhmatia et al. (17). Before the tooth extraction procedure, the right lower central incisor was sliced every three days up to twice in a row. The retention of the periodontal ligament was cut away at the gingival edge using a diamond disk bur to make tooth extraction easier. After cutting the teeth, the right lower central incisor was extracted in a horizontal direction along the tooth axis in the third phase, and the tooth was gently withdrawn using a needle holder with a controlled movement (Figure 5).

Decapitation was carried out by administering a lethal dose of anesthetics. The rat right lower jaw was taken and put in a solution of formalin buffer fixation to make histological preparations. The post-extraction tooth socket was filled with IPRF, HA, or HA-IPRF in the treatment group using a micropipet, whereas the post-extraction tooth socket was kept unfilled in the control group. Following tooth extraction, rats received 0.3 ml each of the painkiller novalgin and the antibiotic gentamicin for 3 days. 4 rats were applied to control, IPRF, HA and HA-IPRF group on day 14 and 28 respectively. A deadly dose of anesthetics was used to induce decapitation.

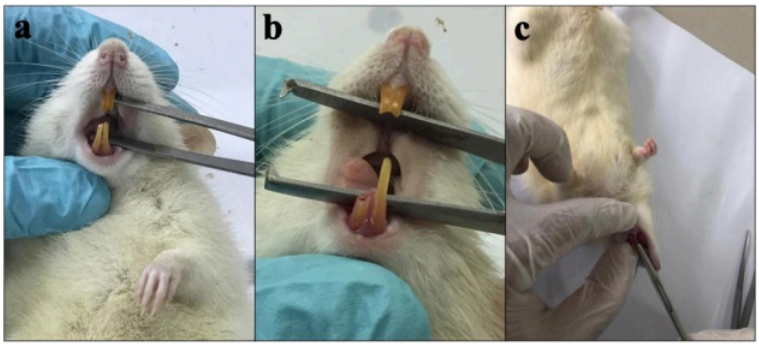


Figure 5. Lower incisor in rat a. before cutting, b. after cutting to the gingival margin, c. rat tooth extraction

2.5 Histological and immunohistochemistry analysis

The preparation was made through the process of decalcification, dehydration, clearing, paraffin infiltration, embedding and 5 µm thick cutting with a transverse direction

parallel to the sagittal plane using microtomes. In immunohistochemical examination, hydration and incubation were performed with rat primary polyclonal antibodies of TRAP (Fine biotech) or ALP (ABclonal technology) or OCN (Fine biotech) overnight. Immunoreactivity of TRAP, ALP and OCN was detected and visualized using secondary antibodies at a room temperature for 1 hour after washing. After reacting with 3,3'-diaminobenzidine, it turned brown as the staining signal. The specimen was observed using a microscope (Olympus Cx33) and camera (Sigma). To assess the formation of new bone in histological preparations, tissue slices were stained with hematoxylin-eosin. All observations were made at 3 different locations representing the proximal, middle and distal parts of the dental socket.

Based on the following scale, the presence of immunopositive cells was determined: (I): 0 = no expression; 1 = weak expression; 2 = medium expression; and 3 = high expression. According to distribution, the presence of immunopositive cells was graded as follows: (p): 1 means brown color 10%; 2 = brown >10%; and 3 = brown >50%. In order to determine the final score of TGF-1 immunopositive cells, the (p) score and (I) score were multiplied using the following classification: A score of 0 indicates no expression, while a score of 1–2 indicates a weakly positive expression, and a score of 3–6 indicates a strongly positive expression.

The new bone formation was examined by measuring the area of woven bone formed in the dental socket using the image raster 3 in the region of interest (ROI) (Figure 6).

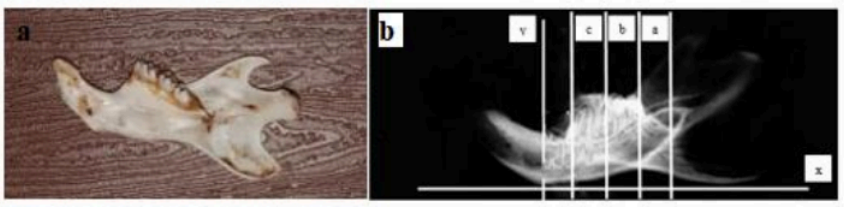


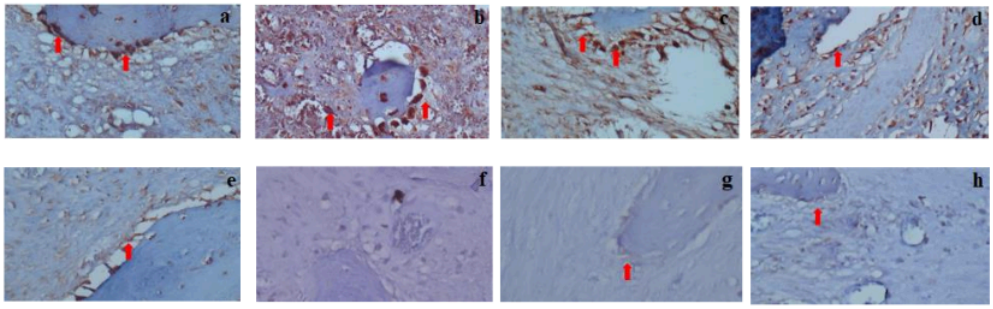
Figure 6. ROI of region a, b and c. The plane (x) is a parallel plane to the mandibular plane and the plane (y) is a perpendicular plane to the mandibular plane

2.6 Statistical analysis

TRAP, ALP, and OCN expression data were performed by the Kruskall-Wallis test and continued by the Mann-Whitney test. New bone formation data were carried out by Saphiro Wilk test to see the distribution of data. If the data are normally distributed, testing is then performed using two-way annova and continued with the Least Significant Difference test. Statistical analysis was done using SPSS 21.

3. Results

3.1 TRAP Expression



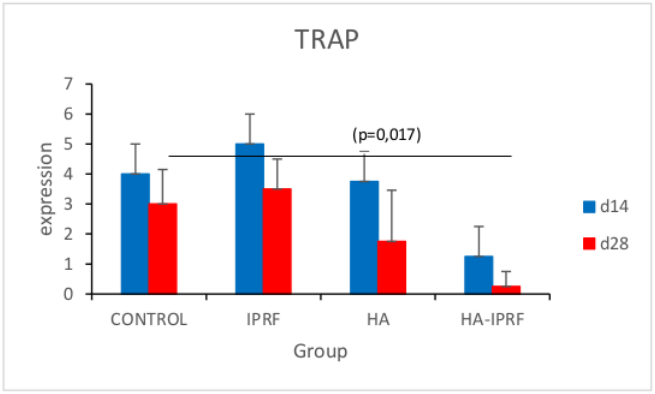
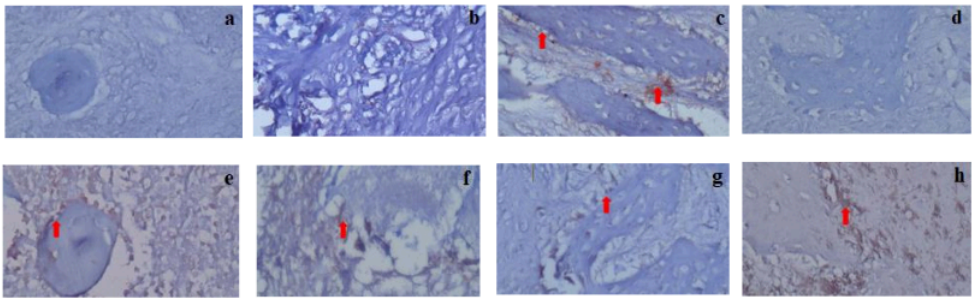


Figure 7. Immunohistochemical description of TRAP expression was detected as brown color (red arrow), 400x magnification, a. control group on day 14, b. control group on day 28, c. group IPRF on day 14, d. group I-PRF on day 28. e. Ha group day on 14, f. Ha group on day 28, g. Ha- IPRF group on day 14, h. Ha- IPRF group on day 28, i. TRAP expression graph in each group. It was seen that the intensity and distribution of TRAP were high in the control group and IPRF group. It was seen that the intensity and distribution of TRAP in the HA group on day 14 was quite high, while in the HA group it was on day 28, and the HA-IPRF group on day 14 and 28 was low. The Kruskall-Wallis test followed by the Mann U Whitney test revealed significant differences between the control group on day 28 and the HA-IPRF group on day 28

3.1 ALP Expression



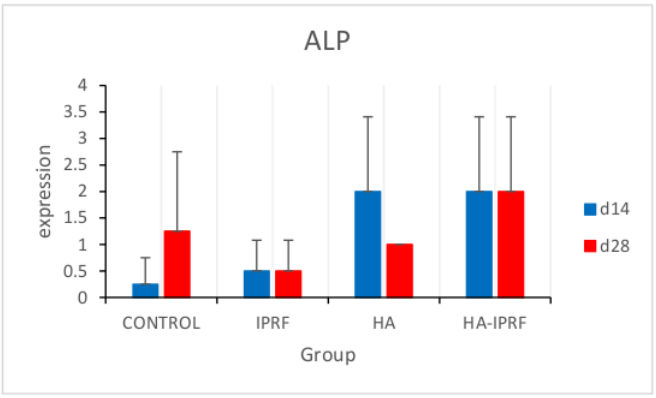
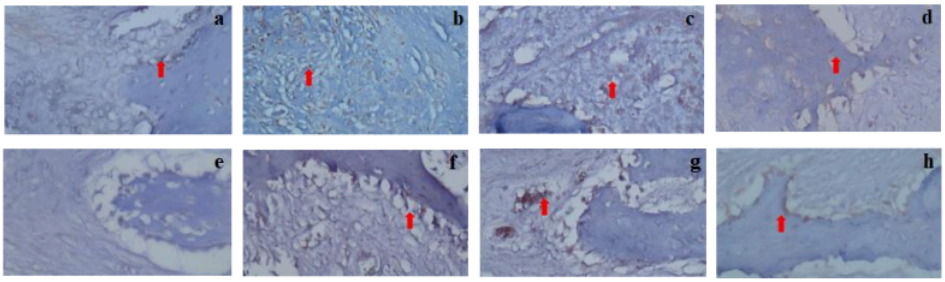


Figure 8. Immunohistochemical description of ALP expression was detected as brown color (red arrow) with 400x magnification, a. control on day 14, b. control on day 28, c. IPRF on day 14, d. IPRF on day 28, Ha on day 14, f. Ha on day 28, g. Ha- IPRF on day 14, h. Ha- IPRF on day 28, i. ALP expression graph in each group. It was seen that the intensity and distribution of ALP in the control group and IPRF were low. The intensity and distribution of ALP in the HA and HA-IPRF groups were found high.

3.1 OCN Expression



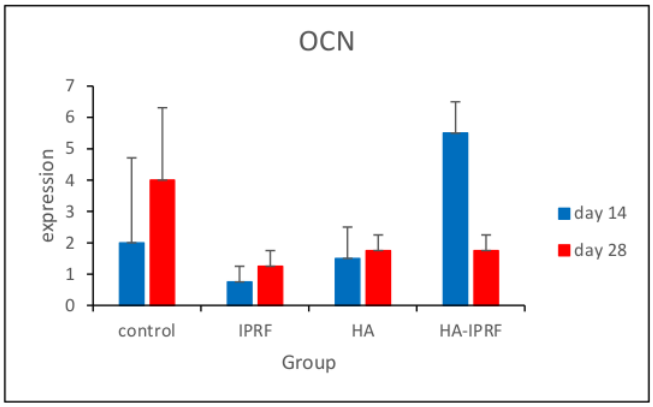
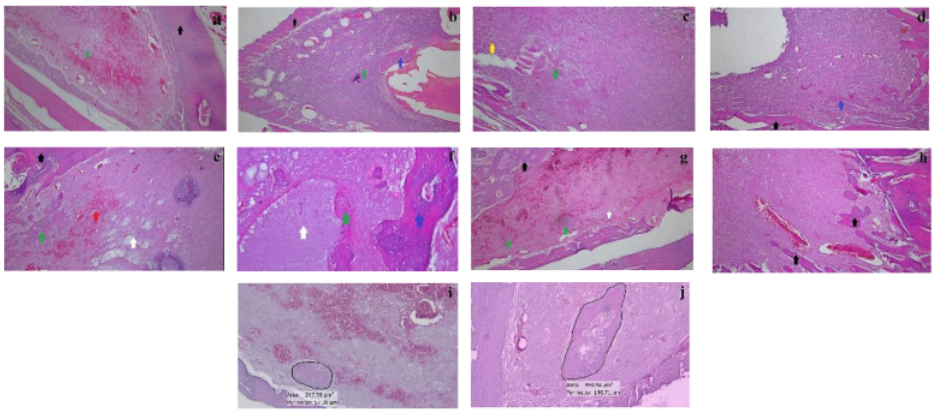


Figure 9. Immunohistochemical description of OCN expression were detected as brown color (red arrow) 400x magnification, a. control group on day 14, b. control group on day 28, c. IPRF group on day 14, d. IPRF group on day 28, e. group Ha on day 14, f. group Ha on day 28, g. group Ha- IPRF on day 14, h. group Ha- IPRF on day 28, i. OCN expression graph in each group. It was seen that the intensity and distribution of OCN on day 28 in control group was high and the IPRF group was moderate. The intensity and distribution of OCN in the HA-IPRF group on day 14 was high and the HA group was moderate.

5.5 New Bone Formation in Post-Extraction Rat Tooth Socket



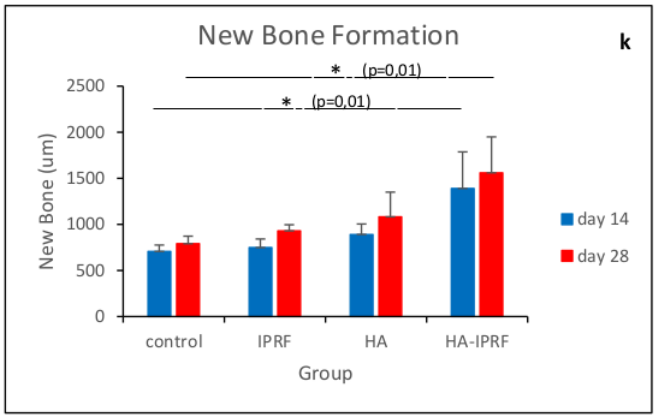


Figure 1. New bone formation description with Hematoxilin-eosin (HE) staining, a. control group on day 14, b. control group on day 28, c. IPRF group on day 14, d. IPRF group on day 28, e. Ha group on day 14, f. Ha group on day 28, g. Ha-IPRF group on day 14, h. Ha- IPRF group on day 28, i. Calculation of new bone area in the control group on day 14, j. calculation of new bone area in the HA-IPRF group on day 14, k. New bone formation graph in each group. It was seen that new bone formation was the highest in the HA-IPRF group and the lowest in the control group. The two-way Annova test followed by the LSD test showed significant differences between the control group on day 14 and HA-IPRF group on day 14, and the control group on day 28 and HA-IPRF group on day 28

5. Discussion

The combination of HA with biological biomaterials has been widely made to improve and accelerate tissue regeneration. A review systematic study by Zaffarin et al. (2021) concluded that nanocrystalline hydroxyapatite was very effective for acting as a delivery system for bone regeneration and binds to proteins, drugs such as antibiotics and other active molecules

thereby increasing osteogenesis in vivo (18). Nanocrystalline hydroxyapatite has bone mineral and good biodegradability properties. It also allows proteins, drugs, and active molecules to attach and slowly be released in bone. Nanocrystalline hydroxyapatite has a particle diameter size of less than 100 nm, which makes it efficiently internalized and can produce an excellent surface area for cell attachment, drugs and other bioactive molecules.This study used nanocrystalline hydroxyapatite made from a pensi shell (*corbicula moltkiana*), and also used IPRF as a combination of nanocrystalline hydroxyapatite to produce a synergistic combination and can increase bone regeneration ability. IPRF is a platelet concentrate with advantages over the previous generation because it is liquid obtained from low speed centrifugation concept, making it easier to mix with other materials (19).

The results of this study proved that the implantation of the HA-IPRF combination can increase and accelerate new bone formation and inhibit alveolar bone reorption after tooth extraction, as indicated by several biomarkers such as decreased expression of TRAP as the indicator of resorption and increased expression of ALP and OCN as that of bone formation.

The results reported that implantation of combined nanocrystalline hydroxyapatite and injectable platelet rich fibrin affected the expression of Tartrate-resistant acid phosphatase in bone healing after tooth extraction where there was a significant difference between the HA-IPRF group and the control group on day 28. The mean difference in TRAP expression between the HA-IPRF group and the control group was also seen on day 14, although it was statistically not significant. The study revealed that TRAP expression on days 14 and 28 was likely to be lower in the group implanted with the HA-IPRF combination compared to the control group where the post-extraction dental socket was not implanted with any material and left to fill with blood clots. It allegedly occured because HA-IPRF implantation controls excessive resorption of alveolar bone as indicated by lower TRAP expression in the HA-IPRF group.

TRAP has been widely known to play a role in signaling the active bone resorption process and a marker of osteoclast activity. A decrease in TRAP expression indicates reduced bone resorption. Alhasimi et al. (2018) suggested similar things in the combination of carbonate hydroxyapatite with aPRF where the combination of these ingredients can improve post-orthodontic treatment stabilization marked by increased osteoblasts, decreased osteoclast and TRAP (20). Ayukawa et al. (2009) disclosed that local application of statins can improve bone healing through osteoclast suppression and increased osteoblasts in rat bone healing areas. In the study simvastatin was found to have affected the decrease in TRAP (21). Boaini et al. (2018) demontrated that coating of strontium hydroxyapatite material on titanium material can inhibit

osteoclasgenesis and osteoclast differentiation shown through decreased number of TRAPs (22).

The results showed high ALP expression on day 14 and 28 in the HA-IPRF combination group compared to the control group although it was not statistically significant. This finding indicates that the HA-IPRF combination biomaterial is very good at stimulating bone growth. ALP is known to be a specific glycoprotein involved in the early stages of osteoblast differentiation and is responsible for the formation of hydroxyapatite crystals in the early days of bone formation (23).

The results of this study pointed out that OCN expression in the HA-IPRF group was higher than that of in the control group on day 14. Hassumi et al. (2018) argued that increased OCN expression indicates bone organization and maturation (24). The trend of increased OCN expression from day 14 to day 28 was also seen in the IPRF group and HA group. OCN expression in the HA-IPRF combination group was lower than in the control group on day 28, which indicates that bone organization and maturation in the HA-IPRF group first occurred on day 14 and reached its peak and then declined on day 28. The results of this study corresponded with those of Damayanti et al. (2020) where osteocalcin expression was higher in the implantation of combined hydroxyapatite and PRF than that of combined hydroxyapatite and platelet rich plasma on day 3, 7 and 14 in rabbit tooth post-extraction healing (25).

Important phenomena were seen in HA group as HA material shows the ability to increase osteogenesis by facilitating osteoblast cells and new blood vessels to develop to form a new bone, among which were found in figure (10.e) where there was growth of woven bone and new blood vessels around the HA material on day 14, while on day 28 (figure 10.f) woven bone was formed which calcifies and mineralizes into mature bone. The results of this study also proved that, when implanted in the socket after rat tooth extraction, nanocrystalline hydroxyapatite was not cytotoxic and did not make excessive immunological reactions. This finding corresponds with the literature review by Bayani et al. (2017) suggesting that nanocrystalline hydroxyapatite is known as a biomaterial that is biocompatible, bioactive, bioresorbable non-toxic, and does not make excessive immunological and inflammatory reactions (26). Nanocrystalline hydroxyapatite also gets involved in suppressing osteoclast work so as to minimize the resorption process, increase osteoblast differentiation and ploriferation, and increase bone formation. However, the results of the study by Rothamel, et al (2008) showed a different finding. The study found that the use of nanocrystalline hydroxyapatite for preservation of alveolar bone after tooth extraction did not generate effects that could prevent changes in alveolar bone dimensions (27).

The results also showed that the IPRF group on day 14 indicates the accumulation of lymphocytes, plasma cells and histiocytes in the post-extraction dental socket (Figure 10.c), allegedly the effects of IPRF application which contains leukocytes and cytokines. IPRF content makes it an anti-bacterial and anti-inflammatory, although the accumulation of lymphocytes, plasma cells and histiocytes was no longer found in the IPRF group on day 28. The results of this study were consistent with those of Varela et al. (2018) which proved that IPRF has a significantly more lymphocyte composition than blood. Furthermore, IPRF is known to contain platelets, leukocytes, type 1 collagen, osteocalcin and growth factor (28).

The results showed that new bone growth was formed in all groups with the highest occurred in the HA-IPRF combination group while the lowest was in the control group on both day 14 and day 28. Figure 10 demontrates the histological description of the dental socket in the control group which shows less new bone growth than the other group on day 14 and the HA-IPRF group shows the most new bone formation while mature bone formation began to appear on day 28 in all groups.

Statistical analysis using two-way Anova indicates significant differences between the groups, because of which it was followed by LSD post hoc test. The test found that there was a significant difference between the HA-IPRF combination group and the control group on both day 14 and day 28. This finding proves that implantation of the HA-IPRF combination can increase new bone growth on day 14 and day 28.

New bone growth upon implantation of the HA-IPRF combination increased because HA is known as a biocompatible, osteoconductive and bioactive material that can foster bone growth while IPRF contains platelet cells, leukocytes and growth factors that can stimulate bone healing. The composition of HA and IPRF was found to contribute considerably in increasing new bone growth where new bone growth in the HA group and IPRF group was higher than in the control group on both day 14 and day 28 although it was not statistically significant. The ability of HA to increase bone regeneration is crucial. Hydroxyapatite will release calcium phosphate when hydroxyapatite is implanted into bone defects. The activity will increase body fluid saturation and precipitate biological apatite in the area. This biological apathy can contain endogenous proteins and act as a matrix for attachment and growth of osteogenic cells (12). IPRF contributes considerably in the high increase in new bone formation by the HA-IPRF group because it contains growth factor, which includes Platelet Derived Growth Factor (PDGF), Transforming Growth Factor (TGF) and Insulin Growth Factor (IGF) involved in the process of osteogenesis (29,30).

This study has successfully disclosed that the combination of HA and IPRF can increase bone regeneration and reduce alveolar bone resorption following rat tooth extraction, therefore it is potential to be material for alveolar-bone preservation. The results agree with those of Mu et al. (2020) which proved that the combination of deproteinized bovine bone mineral with IPRF accelerated vascular formation, bone remodeling and replacement of bone graft material with new bone in the early stages of healing, despite that the combination of materials did not show an increase in bone volume at the long-term healing stage (31). The study by Wang et al. (2021) proved that the combination of particulate bone substitute (Bio-oss) with IPRF has a positive effect on the thickness of the labial part of hard tissue in the coronal implant 6 months postoperatively (30). Kyyak et al. (2021) also revealed that the combination of bovine bone substitute materials with IPRF increases the viability and metabolic activity of human osteoblasts, increases the expression of ALP, BMP-2 at the initial level and OCN at the final level in vitro (32). Mallappa et al., (2022) disclosed that the combination of nanocrystalline hydroxyapatite, aPRF and IPRF materials shows good clinical and radiological results in the treatment of intrabone periodontal defects in humans compared to the single use of nanocrystalline hydroxyapatite (33).

The results of this study have a number of limitations. First, cutting preparations on histological and immunohistochemical observations must be done in an accurate site because the error in cutting site will produce differences in data results, therefore, DNA isolation techniques can be an alternative. Second, the study period consists of day 14 and day 28 only. As a result, the healing process can only be assessed in both periods while the healing process took place throughout the study, and some healing biomarkers can reach peak fluctuations in different periods. Observations were not done until the complete recovery of post-extraction tooth socket.

6. Conclusion

The HA-IPRF combination can surpress alveolar bone resorption marked by decreased TRAP expression, and can increase bone growth as indicated by an increase of ALP expression, OCN and new bone formation. HA-IPRF implantation is feasible to be bone graft material for alveolar bone preservation because it can potentially reduce alveolar bone resorption and increase or accelerate new bone formation.

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8. References

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