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Research Report

Hydroxyapatite combined with hyaluronic acid metronidazole gel increased the quantity of osteoblasts in the alveolar bone wistar rat

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ABSTRACT

Background: *Bone graft material have been used extensively in bone healing and periodontal treatment. Alloplast such as hydroxyapatite are frequently used to repair and reconstruct bone defects. By merely applying hydroxyapatite for the treatment of bone is not fully effective yet to produce new bone regeneration. Locally applied high molecular hyaluronic acid (HA) has been shown to stimulate differentiation and migration of mesenchymal cells. Recent studies on regenerative surgical procedures indicate that reduction of bacterial burden at the wound site may improve the clinical outcome of regenerative therapy. Metronidazole has the greatest bacteriostatic effect. A clinical application of HA metronidazole gels during the surgical therapy may reduce the bacterial contamination of surgical wound site.* ***Purpose:*** *The purpose of this study was to examine the effect of combination hydroxyapatite and HA metronidazole gel 1% on osteoblast cell number after wound healing process in the wistar rats incisor tooth extraction socket.* ***Method:*** *Twenty seven wistar rats were divided randomly into 3 groups. The first group consisted of wistar rats given hydroxyapatite were subjected to the mandibular incisor extraction socket. The second group were given hydroxyapatite combined with HA metronidazole gel 1%. The control group were filled with blood. Wistar rats were euthanized on day 14 and then preparation for histological examination was stained using hematoxylin-eosin and then the numbers of the osteoblasts were calculated.* ***Result:*** *The differences in each group were tested by one way Anova test (α=0.05). The numbers of osteoblasts in each group had a significant difference ($p<0.05$): the highest numbers of osteoblasts were found in the group that was given hydroxyapatite combined with HA metronidazole, followed by the lower numbers of osteoblasts in the hydroxyapatite group and the lowest numbers of osteoblasts were found in the control group.* ***Conclusion:*** *Adjunctive application of HA metronidazole to hydroxyapatite after tooth extraction increase the number of osteoblast in the treatment of bone defects on a wistar rat model.*

Keywords: *hydroxyapatite; hyaluronic acid metronidazole; osteoblast*

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INTRODUCTION

Bone augmentation techniques are commonly employed in dentistry. The role of bone graft materials in regenerative procedures is based on that osteogenic, osteoinductive or osteoconductive potential. Alloplast include hydroxyapatite, collagen-based matrices, calcium sulfate, and tri-calcium phosphate is a synthetic body material that has ostoeconductive capacity. However, hydroxyapatite is not fully effective yet to produce new bone regeneration. Treatment of bone defects by using hydroxyapatite alone cannot be maximized in new bone forming. Indeed, hydroxyapatite were found enhance osteoblast differentiation, but combined hydroxyapatite collagen or growth factor together were shown to accelerate osteogenesis.[1,2] One of many approaches of tissue engineering is to create a device of similar mechanical and biological properties to the one of the substituted tissue. The use of regenerative materials and antibacteria which is used in conjunction with hydroxyapatite can increase

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the accomplishment of bone augmentation. It is expected that the use of regenerative materials such as hyaluronic acid, and antibacteria such as metronidazole which is used in conjunction with hydroxyapatite can increase the accomplishment of the bone healing.

Bone healing is a complex process that involves local and systemic organic activity and osteoblast are some of the cells directly involved in this mechanism. There are three phases in bone healing including reactive phase (phase inflammation and granulation tissue formation), reparative phase (cartilage callus formation and deposition of lamellar bone) and remodeling phase (formation of bone contour). Osteoblasts plays an important role in the mineralization process. Osteoblast proliferating from the pre-existing bone beneath the calcified cartilage, and in conjunction with angiogenic activity, form bone on the calcified cartilage stratum. This bone formation process is endochondral ossification. Small bone defects, such as socket after tooth extraction, heal by direct synthesis of new bone to fill the defect. After extraction no bone formation occurred for the first week. At 8 days, new bone formation was noted throughout the alveolar bone, particularly under the wall but not on the surface of the bone lining the extraction socket. At 10 days, bone formation was noted on the surface of the socket wall. At 12 days, new bone formation continued along the socket wall and in the trabecular spaces surrounding the extraction site. Osteoblasts regulates calcium and phosphate concentrations. In addition osteoblasts also express alkaline phosphatase in high quantities to the plasma membrane. Osteoblasts as secretory metabolically active, producing a number of BMP-2, BMP-7 and growth factor. Osteoblasts also express RANKL and OPG expression products of osteoblasts occurs during bone remodeling.[3,4]

In the early stages of inflammation, the tissues will contain a large amount of Hyaluronic acid which is much needed for the process of bone regeneration. Hyaluronic acid (HA) is hyaluronan/hyaluronate which is a non-sulfated polysaccharide component from the glycosaminoglycan group with high molecular weight (10,000–10,000,000 Da). HA serves to accelerate the process of regeneration by way of chemotaxis, proliferation and differentiation of mesenchymal cells and share the role as bone induction with osteogenic substance such as bone morphogenetic protein-2 (BMP-2) and osteopontin.[4,5] HA is one of the essential components of extracellular matrix, which plays a predominant role in tissue morphogenesis, cell migration, differentiation, and adhesion which will contribute bone healing properties. In the medical world apart from being used for therapeutic in dentistry, hyaluronic acid is also used for the treatment of the skin, joints, hair and eyes. [6]

Recent studies on regenerative surgical procedures indicate that reduction of bacterial burden at the wound site may improve the clinical outcome of regenerative therapy. Metronidazole has the greatest bacteriostatic effect, particularly on *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Prevotella oris* and *Staphylococcus aureus* strains, which are commonly found in oral lesions. A clinical application of HA metronidazole gels during the surgical therapy may reduce the bacterial contamination of surgical wound site, thereby, lessening the risk of postsurgical infection and promoting more predictable regeneration.[7] Considering that, no previous study exists on the use of hydroxyapatite combined with HA metronidazole as adjunctive to grafting process. The purpose of this study was to examine the effect of combination hydroxyapatite and HA metronidazole gel on osteoblast cell number after wound healing process in alveolar bone wistar rat.

MATERIALS AND METHODS

The study is a laboratory experimental research with randomized post test only control group design. The sample used 27 wistar rats aged 8-16 weeks were divided randomly into 3 groups which had 9 wistar rat each. The study protocol had been approved by Ethical Committee of Faculty of Dental Medicine Universitas Airlangga, Indonesia (No. 197/KKEPK.FKG/XI/2015). The first group consisted of wistar rats given hydroxyapatite were subjected to the mandibular incisor extraction socket. The second group consisted of wistar rats that were given hydroxyapatite combined with HA metronidazole on the mandibular incisor extraction socket. The third group as control which the mandibular incisor extraction socket was filled with blood. The materials used in the present study were HA metronidazole with a concentration 1% in a gel form and hydroxyapatite. HA metronidazole gel 0.5 mg was mixed with hydroxyapatite 0.5 mg in the same ratio 1: 1 were applied into the socket of dental extraction. The extraction cavity was then covered with coronally repositioned flap, sutured and compressed with the moist gauze. Animals were placed on a soft diet for two days post surgically.

Prior to the treatment, the wistar male rats were acclimatized for one week. The rats were maintained in a 40 cm x 30 cm x 15 cm size plastic cage. They were given the same food and drink during the adjustment period. The plastic cage was covered with woven wire, and the cage floor was covered with husk. The cage was placed in an isolated room with good ventilation and natural lighting. The rats was decaputated 14 days after application by injection of ketamine 50 mg/kg. Immediately after sacrifice, animals' jaws were carefully dissected, and the experimental teeth with their surrounding bone tissue were block-sectioned using electric surgical saw. Blocks were immediately fixed in a 70% buffered formalin solution to prevent tissue changes so they do not decay, or harden, increase the refractive index of various tissue components and increase tissue affinity against paint materials. After complete decalcification, blocks were routinely processed and embedded in paraffin. Six mμ sections were cut in a buccolingual direction, mounted on glass slides, deparaffinized, hydrated and stained. After the 48 hours, the fixative was replaced with a new one and the tissues

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were cut much smaller so that the fixation could penetrate evenly into the tissues. In the second stage, the fixation was left in the solution for 48 hours. After fixation the tissues were rinsed with running water for 6-9 hours, and then put in a HNO3 5% decalcification solution for 1 hour. On the next stage, hematoxylin and eosin (H & E) stain was performed on the preparations for histology. Histomorphometric analysis was carried out on the healing sites. In each HE stained slide, three digital images were captured at (400) magnification. Each group was observed with an OLYMPUS BX41 series microscope which is equipped with a D70 digital camera using the OLYSIA software. The intensity of the color in the osteoblast areas could be seen as a quantitative value. This research data was analysed by one way Anova statistical test and continued with Tukey HSD.

RESULTS

Osteoblast obseved were found in the apical third of the tooth socket. Histological examination of all treated cavities sites demonstrated newly osteoblast of implemented materials. Figure 1 shows histological imaged that observed under the microscope.

Normality and homogenity tests were carried out first to obtain data of the research. The Kolmogorov-Smirnov test was applied for normality test while the Levene test was used for homogeneity test. Results of the Kolmogorov-Smirnov test denoted the probability of each treatment group which were bigger than 0.05 ($p>0.05$). The values mean that all the data were taken from the symmetrical or normal distributions. In the Levene test result on osteoblasts sig = 0.977 means $p>0.05$ data was homogeneous. The data acquired was known normally and distributed homogeneously, next step one way Anova test was to carry out to determine the differences in the number of osteoblasts in all the three treatment groups. One way Anova test continued Tukey HSD test showed that the probability figure obtained was 0,000 ($p<0.05$). Therefore the average of osteoblast cell on each group was a significant difference (Table 1 and 2).

DISCUSSION

Hydroxyapatite has been used for a variety of biomedical applications, including matrices for drug release control and bone tissue engineering materials. Hydroxyapatite is chemically similar to the inorganic component of bone matrix–a very complex tissue with general formula $Ca_{10}(OH)_2(PO_4)_6$. Hydroxyapatite exhibits strong affinity to host hard tissues. Chemical bonding with the host tissue offers hydroxyapatite a greater advantage in clinical applications compared to most other bone substitutes such as allografts or metallic implants. The main advantages of hydroxyapatite are its biocompatibility, slow biodegradability in situ, and good osteoconductive capabilities. Hydroxyapatite exhibits excellent biocompatibility with soft tissues such as skin, muscle and gums and also has been widely used to repair hard tissues. Common uses include bone repair, bone augmentation, as well as coating of implants or acting as fillers in bone or teeth.[8,9]

Bone healing includes a series of highly reproducible and rigidly controlled biologic events (inflammation, granulation tissue formation, epithelium formation and tissue remodelling) which begin with chemo attraction of cells that accumulate and debride the injured tissue, foreign material, and microbial cells. HA possesses various

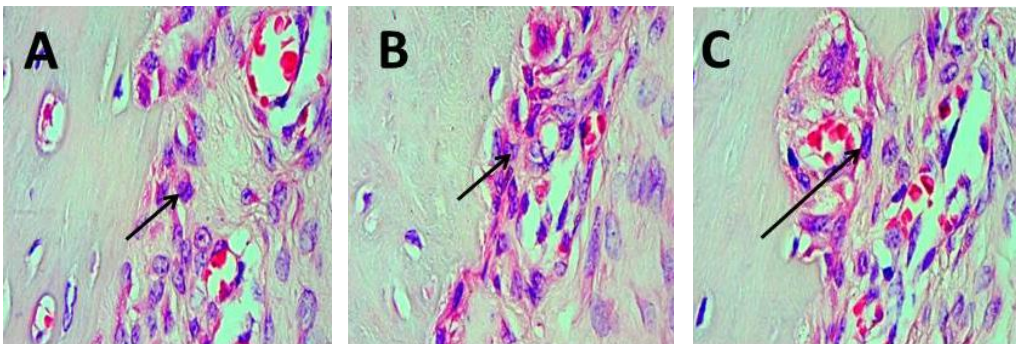

Figure 1. Alveolar bone tissue preparations showing the existence of osteoblast quantity on (A) hydroxyapatite group; (B) hydroxyapatite combined with HA metronidazole group; and (C) control group.

Table 1. The mean and standard deviation of the number of osteoblasts cells

No.	Group	N	$\overline{X}$ ±SD	Sig
1.	Hydroxyapatite	9	14.00 ± 2.739	0.00
2.	Hydroxyapatite + HA metronidazole	9	19.00 ± 1.500	0.00
3.	Controlled	9	3.56 ±1.236	0.00

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Table 2. Significancy of Tukey HSD test

Tukey HSD Multiple Comparisons					
Dependent Variable	(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.
Osteoblast	HA	Hap + HA	-8,222*	,651	,000
		control	7,222*	,651	,000
	HAp + HA	HA	8,222*	,651	,000
		control	15,444*	,651	,000
	control	HAp	-7,222*	,651	,000
		HAp + HA	-15,444*	,651	,000

*. The mean difference is significant at the 0.05 level. HAp : Hydroxypatite

physiological and structural functions, which include cellular and extracellular interactions with growth factors and regulation of the osmotic pressure and tissue lubrication. All these functions help in maintaining the structural and homeostatic integrity of the tissue. These biopolymers are completely biodegradable and support the growth of fibroblasts, osteoblast, chondrocytes and mesenchymal stem cells. Many proteins play a role in binding HA, namely, fibrinogen, fibrin, fibronectin, and collagen. All these molecules play an important role in wound healing.[10] The mechanisms by which HA promote bone formation were attributed to some actions. HA accelerated bone regeneration by means of chemotaxis, proliferation and successive differentiation of mesenchymal cells. HA increased alkaline phosphatase and hence stimulate cell mineralization. HA allowed the early deposition of osteoid tissue by providing a scaffold on which osteoprogenitor cell attached and so stimulated osteoblastic differentiation. Furthermore, the anti-inflammatory ability of exogenous HA may reduce the postsurgical inflammation and bacterial contamination when added to the surgical site and finally improve the surgical outcomes. Through recognition of its viscoelastic nature, HA can influence the cell functions that modify the surrounding cellular and the extracellular micro and macro environments. The viscoelastic properties of the material may slow the penetration of viruses, and bacteria, a feature of particular interest in the treatment of periodontal diseases.[11]

In this study a good response is seen in the use of a combination of hydroxyapatite and HA metronidazole compared with merely using hydroxyapatite. It is seen from the increasing number of osteoblasts in the healing process of the male wistar rats' sockets, because HA is distributed by polysaccharide components of the extracellular matrix of connective tissue and bone marrow which has a function, among others, to facilitate cell migration, cell movement, cell interaction, cell matrix adhesion, differentiation during the formation and tissue repair and regeneration. HA acting on mineralized tissue and non-mineralized is found in the extracellular space of the entire tissues and synthesized in the cellular plasma membrane. HA serves to accelerate the process of regeneration by way of chemotaxis, proliferation and differentiation of mesenchymal cells and share the role as a bone induction with osteogenic substance such as bone morphogenetic protein-2 and osteopontin.[12] In vitro studies HA is known to have a role in improving the formation of osteoblasts of the bone by increasing differentiation and migration of mesenchymal cells.[13] HA shares a role in inducing bone with the osteogenic substrate such as calcitonin and bone morphogenic protein (BMP). BMP are the growth factors generally known to stimulate new bone formation.[12-14] HA is recognized by specific cell receptor CD44 acting as selective and protective quote around cell membrane. CD 44 can exist in numerous isoforms. HA binding properties of CD44 are determined by the isoform and the cell type on which it is expressed. HA participates in tissue repair and wound healing and is used topically as anti inflammatory and anti-oedematous agent. The anti-inflammatory effect may be due to action of exogenous hyaluronan as a scavenger by draining prostaglandins, metalloproteinases and other bioactive molecules increases proliferation, metabolism and cell migration, thereby accelerating bone healing.[15,16]

Recent studies on regenerative surgical procedures indicate that reduction of bacterial burden at the wound site may improve the clinical outcome of regenerative therapy. The most common chemotherapeutic agents are antimicrobials and anti-inflammatory drugs. They are administered either systemically or topically. Topical antimicrobial agents for the treatment of bacterial anaerob include metronidazole. Metronidazole is a derivative of nitroimidazole, it has antiprotozoal and antibacterial action. The mechanism of action of metronidazole is in biochemical reconstruction of its 5-nitrogroup by intracellular transport proteins of anaerobic bacteria and protozoa. Recovered 5-nitrogroup of metronidazole is reacted with the DNA of microorganism cell by inhibiting the synthesis of nucleic acids, which leads to the death of the bacteria. [7]

Tissue damage or impaired bone healing causes bacterial infection is a secondary result. Acute alveolar osteitis is a relatively common complication of the routine extraction of teeth. Antibacterial strategies have shown more promise. Preoperative good results have been obtained with topical antibiotics including clindamycin, metronidazole and tetracycline.[7]

In the research, metronidazole was choosed as tropical antibiotic because its good safety profile, low risk of allergy and effectiveness against pathogens which cause oral

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infections. Metronidazole is particularly suitable due to its restricted spectrum of activity against obligate anaerobes and its limited side-effects. Metronidazole is classified in the WHO Essential Medicines List as antiamoebic, antigiardiasis, and antibacterial. Since infection control is one modifiable factor, which can be controlled by the clinician, to so attempts have been made to incorporate metronidazole which is an antimicrobial drug along with hyaluronic acid gel to prevent bacterial contamination to be utilized as a graft material. Gel with metronidazole improved wound healing during the 1st week after surgery owing to maximum growth factor concentration and antibiotic action for the first 5-7 days. It was also observed that significant improvement in bone regeneration (reduction in defect size) occurred over the entire course of healing as evident by the rapid and more complete bone growth.[17] Probably, the control of microbial population has resulted in successful healing and a good regeneration response.

For the research, HA was used as carrier of metronidazole because HA are promising in drug delivery applications. HA affects the permeability of tissues and the transfer of other medical substances. HA plays invaluable role not only as an independent medicine, but also as an instrument of slow transfer of other therapeutic agents to the tissues, providing also their controlled release. Biologically active components may be covalently or non-covalently bound to HA by varying of the concentration. It is possible to control the rate of its degradation or diffusion and, therefore, the speed of delivery of a medicine to the tissues. HA creates a depot of a medicine in the place of application and gradually collapsing frees a medicine, improving its pharmacological profile and preventing the development of possible adverse reactions. Viscoelastic properties and shear thinning character was strongly dependent on pH. Structured systems were obtained at pH 3, with an increase of several orders of magnitude in zero-shear viscosity values.[18]

The most recent research shows that HA assists the process of repair in both the hard and soft tissues. HA has a high capacity of water holding-one molecule of hyaluronic acid binds 200-300 water molecules. Together with other proteoglycans hyaluronic acid is a member of the extracellular matrix. Due to its physical and chemical properties such as high viscosity contributes to the manifestation of numerous functions of connective tissue. HA affects the permeability of tissues and the transfer of other medical substances. A clinical application of HA membranes, gels, and sponges during the surgical therapy may reduce the bacterial contamination of surgical wound site, thereby, lessening the risk of postsurgical infection and promoting more predictable regeneration.[19,20] In conclusion adjunctive application of HA metronidazole to hydroxyapatite after tooth extraction increase the number of osteoblast in the treatment of bone defects on a wistar rat model.

REFERENCES

1. Elkaragy A. Alveolar sockets preservation using hydroxyapatite/ beta tricalcium phosphate with hyaluronic acid (Histomorphometric study). J Am Sci 2013; 9(1): 556-63.
2. Joung YK, Heo JH, Park KM, Park KD. Controlled release of growth factors from core-shell structured PLGS microfibers for tissue engineering. Biomat Res 2011; 15: 78.
3. Yun YR, Kim HW, Jang JH. Application of growth factors in tissue regeneration. Biomaterial Research 2013; 17: 133.
4. Radhi HI, Ghaban NM. Evaluation the effect of hyaluronic acid on bone healing process in rabbits (Immunohistochemical study for TGF-β. J Bagh College Dentistry 2015; 27: 111-6.
5. Necas J, Bartosikova L, Brauner P, Kolar J. Hyaluronic acid (hyaluronan): a review. Veterinarni Medicina 2008; 53(8): 397-411.
6. Dahiya P, Kamal P. Hyaluronic acid: a boon in periodontal therapy. N Am J Med Sci 2013; 5(5): 309–15.
7. Zamani M, Morshed M, Varshosaz J, Jannesari M. Controlled release of metronidazole benzoate from poly(ε-caprolactone) electrospun nanofibers for periodontal diseases. Eur J Pharm Biopharm 2010; 75(2): 179-85.
8. Haider A, Gupta KC, Kang IK. Morphological effects of HA on the cell compatibility of electrospun HA/PLGA composite nanofiber scaffolds. Biomed Res Int 2014; 7: 306-9.
9. Krishnamurty G. A review on hydroxyapatite-based scaffolds as a potential bone graft substitute for bone tissue engineering applications. Jummec 2013; 16: 1-6.
10. Bezzerra B, Brazao MA, Casati MZ, Sallum EA, Sallum AW. Association of hyaluronic acid with a collagen scaffold may improve bone healing in critical-size bone defects. Clin Oral Implants Res 2012; 23(8): 938-42.
11. Gontiya G, Galgali SR. Effect of hyaluronan on periodontitis: A clinical and histological study. J Indian Soc Periodontol 2012; 16(2): 184-92.
12. Nie H, Wang CH. Fabrication and characterization of PLGA/HAp composite scaffolds for delivery of BMP-2 plasmid DNA. J Control Release 2007; 120(1-2) :111-21.
13. Patterson J, Siewa R, Herringb SW, Linc ASP, Guldbergc R, Staytona PS. Hyaluronic acid hydrogels with controlled degradation properties for oriented bone regeneration. Biomaterials 2010; 31(26): 6772–81.
14. Joung YK, Heo JH, Park KM, Park KD. Controlled release of growth factors from core-shell structured PLGS microfibers for tissue engineering. Biomaterial Research 2011; 15: 78.
15. Park HK, Lee SJ, Jong-Suk , Lee SG, Jeong Y, Lee HC. Smart nanoparticles based on hyaluronic acid for redox-responsive and cd44 receptor-mediated targeting of tumor. Nanoscale Research Letters 2015; 10: 288.
16. Jordan A, Racine RR, Hennig JP, Lokeshwar VB. The role of CD44 in disease pathophysiology and targeted treatment. Front Immunol 2015; 6: 182.
17. Tripthi PS, Girieh R, Reethi B. PRF gel as an medium for local delivery of metronidazole. IJHS 2014; 1: 113-22.
18. Takeda K, Sakai N, Shiba H, Kurihara, Mizuno H, Hiroaki. Characteristics of high-molecular- weight hyaluronic acid as a brain derived neurotrophic factor scaffold in periodontal tissue regeneration. Tissue Eng Part A 2011; 17: 955–67.
19. Baldini A DDS, Zaffe D MD, Nicolini G. Bone-defects healing by high-molecular hyaluronic acid: preliminary results. Ann Stomatol (Roma) 2010; 1(1): 2-7.
20. Francesco B, Enrico B, Roberto B, Carlo C. Treatment of infrabony periodontal defects using a resorbable biopolymer of hyaluronic acid. A randomized clinical trial. Quintessence Int 2013; 44: 231–40.

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