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Research Report

Biocompatibility and osteoconductivity of injectable bone xenograft, hydroxyapatite and hydroxyapatite-chitosan on osteoblast culture

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ABSTRACT

Background: *Bone graft in the form of injectable paste gives several advantages over the powder form as it could be placed in the defect area that has limited accessibility.* ***Purpose:*** *The purpose of this study was to assess biocompatibility and osteoconductivity of an injectable bone xenograft (IBX), injectable hydroxyapatite (IHA) and injectable hydroxyapatite-chitosan (IHA-C) on osteoblastic cell line (MG-63).* ***Methods:*** *Three concentrations (0.25%, 0.5% and 1.0%) of IBX, IHA and IHA-C were supplemented with DMEM culture medium. The viability cells were measured by MTT assay 4 hour after incubation. ALP activity was measured at day 1, 3, 5 and 7. Calcium deposition was tested at day 3 and day 7 by means of Von Kossa staining.* ***Results:*** *MTT assay showed that the viability cells of all the test groups were above 100% compared to the control group. The cell viability of the 0.25% IHA paste was significantly higher (115.02% ± 4.37%, $p < 0.05$) compared with IBX paste and IHA-C in all concentrations tested. The highest level of ALP secretion of all test groups was found on the fifth day of exposure. The highest level of ALP in the IBX paste group was 0.25% concentration while the highest level of ALP in the IHA-C and IHA paste group was 1% and 0.25%, respectively. In addition, the highest calcium deposition was shown on IHA 1% at day 7 ($p > 0.05$).* ***Conclusion:*** *It was suggested that adequate biocompatibility and osteoconductivity was evident for all injectable pastes tested.*

Key words: *Injectable bone xenograft, injectable hydroxyapatite, injectable hydroxyapatite-chitosan, osteoblast*

ABSTRAK

Latar belakang: *Bahan tandur tulang dalam bentuk pasta injeksi memiliki kelebihan dibandingkan bila bahan tersebut berupa bubuk, karena lebih mudah diaplikasikan pada daerah yang sulit dijangkau.* ***Tujuan:*** *Penelitian ini bertujuan untuk mengamati sifat biokompatibilitas dan osteokonduktifitas biomaterial tandur tulang dalam bentuk injectable bone xenograft (IBX), injectable hydroxyapatite (IHA) dan injectable hydroxyapatite-chitosan (IHA-C) pada galur sel osteoblas (MG-63).* ***Metode:*** *Bahan tandur tulang IBX, IHA and IHA-C masing-masing dengan konsentrasi 0,25%, 0,5% dan 1,0% dipaparkan dalam larutan medium kultur sel DMEM yang telah disebari sel MG 63. Selanjutnya setelah 4 jam inkubasi maka viabilitas sel diukur dengan cara uji MTT, sedangkan aktifitas fosfatase alkali (ALP) diukur pada hari ke-1 (24 jam), hari ke-3, 5 dan 7. Deposisi kalsium diukur pada hari ke-3 dan ke-7 dengan metoda pewarnaan Von Kossa.* ***Hasil:*** *Uji MTT menunjukkan bahwa pemberian semua jenis bahan pasta injeksi tandur tulang meningkat di atas 100% dibandingkan kontrol. Viabilitas sel pada pemberian 0,25% pasta IHA tampak paling tinggi dibandingkan pasta IBX dan IHA-C pada semua konsentrasi yang diuji. Sekresi ALP tertinggi pada semua kelompok eksperimen terjadi pada hari ke lima setelah paparan bahan injeksi tandur tulang. Sekresi ALP tertinggi pada tiap jenis pasta terjadi pada pemberian IBX 0,25%, IHA-C 1% dan IHA 0.25%. Sedangkan deposisi kalsium tertinggi terjadi pada pemberian 1% IHA setelah 7 hari kultur sel.* ***Kesimpulan:*** *Semua bahan injeksi tandur tulang yang diuji pada kultur osteoblas bersifat biokompatibel dan berpotensi osteokonduktif.*

Kata kunci: *Injectable bone xenograft, injectable hydroxyapatite, injectable hydroxyapatite-chitosan, osteoblas*

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INTRODUCTION

Bone defect is a common finding in oral maxillofacial and orthopedic surgeries. This condition resulted in impair bone function as a structural support. The primary causes of bone defect in the oral maxillofacial region are periodontal disease and tooth loss. Alveolar bone loss in periodontal disease is triggered through immune responses, resulting from inflammatory reaction directed against periodontopathic bacteria.[1] When tooth is extracted and no dental implant is placed, there is no more direct loading that is normally carried by the dentition and transferred through the periodontal ligament to the alveolar bone. This condition leads to a decrease in osteoblasts activity and an increase in osteoclasts activity as mechanical loading play a major role on maintaining bone mass.[2,3] The increase in osteoclasts activity will results in bone resorbtion and subsequently the formation of bone defect that impair the structure as well as the function of bone.

Treatment of bone reconstruction is therefore necessary to reform the volume and density of the bone, thus maintaining normal bone function. Bone reconstruction technique is normally carried out using bone graft.[4] Bone grafts are extensively used for oral and maxillofacial applications including treatment of fractures and non-unions, replenishment of bone loss resulting from tooth extraction, periodontal diseases or tumor.[5] Which material is the most appropriate to restore bone volume is a subject of debate. Autogenous bone graft whereby the bone donor derives from patient's own body is considered the gold standard due to its biological characteristic, providing both organic and inorganic matrices, biological signals and viable bone cells.[5,6] However, in a case of large bone defect, a large quantity of autogenous bone is required to reconstruct adequate bone volume. The procedure gives rise to patient's morbidity. Several alternative materials have been used to overcome the problem such as allograft, xenograft and alloplast.[7-9] The advantage of using bone substitute material is the absence of additional surgery. The utilization of these materials as bone grafts may reduce the need for autogenous bone graft, which mostly available in a limited volume.

One of strategies for tissue engineering is the transplantation of cells that have been expanded in vitro by biodegradable scaffold. Ideal scaffold should possess characteristics such as; biocompatible, biodegradable, high surface area/volume ratio that could support the attachment, proliferation and differentiation of cells to develop the desired tissue.[10] A variety of carriers and matrices have been used for bone regeneration including bone autograft, bone allograft, natural component such as collagen membrane and synthetic carrier such as bioglass, β-TCP.[10,11] Chitosan is a compound that consists of co-polymer glucosamine and N-acetylglucosamine that is currently used for food industry. Chitosan has a good biocompatibility property, degradable by enzyme to become oligosaccharide that can be easily absorbed, forming an insoluble complex with connective tissue such as collagen and glycosaminoglycans to become porous scaffold, film or particle.[12] Chitosan has a porous interconnected structure that makes this biomolecule suitable for scaffolding material especially for tissue engineering.[11] Xenograft is a graft of tissue taken from a donor of one species and grafted into a recipient of another species. The most common sources of xenogenic grafts are bovine.[7-9] This graft acts as scaffold to support the growth of the new tissue and will be replaced with the tissue from host with some reported its minor osteoinductive property. Hydroxyapatite (HA) is often used as a bone substitute material due to its osteoconductive and biocompatible properties allowing the integration with the host bone.[13] The combination of HA and chitosan in the form of injectable, porous and biodegradable structures seem to be an interesting route to promote localized bone regeneration.[14]

Biocompatibility and the absence of contagious substance in the graft are the importants characteristic for ideal bone graft. Bone graft in the form of injectable paste gives several advantages as it could be placed in the defect area that had limited accessibility, allowing the cavity filled with the biomaterial in a homogenous manner accordingly.

This research focus on assessing the biocompatibility and osteoconductivity of three different pastes, namely injectable hydroxyapatite paste (IHA), injectable hydroxyapatite chitosan (IHA-C) paste, and injectable bone xenograft paste (IBX) as prospective scaffolding materials to be used for bone tissue engineering. All tested were performed in the osteoblastic cell line (MG-63). The testing of the toxic effect was carried out with the MTT assay, whereas osteoconductivity property was evaluated with the alkaline phosphatase and Von Kossa staining for calcium deposition.

MATERIALS AND METHODS

The study was conducted at Oral Biology Laboratory, Faculty of Dentistry University of Indonesia. and it was design as an in vitro experiment. The experiments were divided into 4 main groups: one control group that did not received any materials as well as three treatment groups that received a) 0.25%, 0.5% and 1.0% of IBX; b) 0.25%, 0.5% and 1.0% of IHA and c) 0.25%, 0.5% and 1.0% IHA-C. Each experiment was perform in 6 replicates. Osteoblast-like MG-63 cells (ATTC No. CRL-1427; a kind give from Prof. Suttatip Kalmolmatyakul, Prince of Sonkla University, Thailand). The cells were cultured up to near-confluence in 75 cm^2 flasks (Nunc) using DMEM, Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, SIGMA), penicillin, 100 IU/ml and 100 pg/streptomycin. Cells were then harvested and seeded at 1×10^5 cells per well (incubated overnight in DMEM with 10% FBS to promote cell attachment at 37° C with 5% CO_2 in air. The cells were then divided into two groups,

a test group, incubated with media containing various concentrations of injectable grafts tested and a control group, incubated only with the media. IBX, IHA-C, and IHA (BATAN, Indonesia) were diluted in the culture medium until 1%, 0.5%, and 0.25% concentration were reached.

To estimate the density of viable cells, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay was conducted.[15] The samples were divided into 1×10^6 cells/ml/well in a 96-well culture plate and were incubated for 24 h. Injectable paste grafts were then added to the media for 4h. The samples were washed by PBS and MTT dye agent (Sigma) was mixed to each well and incubated for another 4h. The absorbance was measured using a microplate reader at the wavelength of 490 nm (Biorad). To obtain the percent of cells viability, the optical density (OD490) of treatment group was devided to the control group.

To measure early osteoblast differentiation, alkaline phosphatase (ALP) activity test was carried out from culture medium by colorimetry.[16] The samples were divided into 1×10^5 cells/ml/well in a 24-well culture plate for 24 h and followed by incubation with injectable paste grafts for 1, 3, 5 and 7 days. ALP substrate solution of P-nitrophenyl phosphate (Sigma) was added to each solution at room temperature for 30 m. The absorbance was measured using a microplate reader at the wave length of 405 nm.

To measure calcium deposition by MG63, van Kossa staining was performed. The samples were divided into $1x10^5$ cells/ml/well in a 6-well culture plate for 24 h and cultured in the media containing various concentrations of bone graft tested for 3 and 7 days. Mineralized nodules were stained with silver nitrate solution according to the von Kossa method as described.[17] The results were statistically analyzed by ANOVA for normal data and Mann Whitney for abnormal data. *P* value of < 0.05 represented a significant difference.

RESULTS

Viability cells measurement using MTT assay showed that all of the test groups compared to control group was above 100% (Table 1). The highest cell viability was found in the group of cells incubated with 0.25% IHA paste (115.02% ± 4.37%, $p < 0.05$). Of all concentrations tested in three different injectable graft pastes, lower concentration of 0.25% demonstrated higher cell viability compared to high concentrations of 0.5% and 1.0% ($p < 0.05$). The highest level of ALP secretion of all test groups occurred on the fifth day of exposure (Table 2). The highest level of ALP was found in the 0.25% concentration of IBX paste group (optical density = 1.8895); whilst the highest level of ALP in the IHA-C and IHA paste group was 1% and 0.25% concentration respectively (optical density = 1.8465; 1.7475). However, the increase was not statistically significant ($p > 0.05$) when compared with the control. In addition, the highest calcium deposition was shown on IHA 1% at day 7 ($p > 0.05$). All injectable pastes increase calcium deposisition compare to the control at day 7 of culture, whereas at day 3 these initial mineralization have occured in lower level (above 1040 spots), (Table 3).

Table 1. Effect of IBX, IHA-C, HA pastes. The viability of MG63 osteblastic cell line according to the MTT assay

Concentration of paste	MG63 cell viability		
	IBX	IHA-C	IHA
1%	104.42%	107.52%	102.37%
0.5%	108.54%	112.82%	100.38%
0.25%	109.21%	115.02%*	109.32%

*The cell viability of the 0.25% IHA paste was significantly higher (115.02% ± 4.37%, $p < 0.05$) compared with IBX paste and IHA-C in all concentrations tested.

DISCUSSION

In vitro culture system was one of the methods commonly used to evaluate the biological responses of biomaterial prior its to be tested on animal model. This system has some avantages over the animal study, as we can directly asses the appropriate cell responses against material to be tested. Here we use osteoblast cell line to evaluate the effects of bone graft pastes on the cells viability, osteogenic biomarker secreted by the cells as well as calcium deposisition. The outcome of the study is expected to be the foundation for the development of bone tissue engineering.

Table 2. The effect of Injectable pastes in alkaline phosphatase level of MG63 osteblastic cell line culture medium

Day	IBX (concentration)			IHA-C (concentration)			IHA (concentration)			Control
	1%	0.5%	0.25%	1%	0.5%	0.25%	1%	0.5%	0.25%	
1	1.1095	1.103	1.5845	1.427	0.7725	1.641	1.4325	0.868	1.651	1.2215
3	1.57	1.621	1.1345	1.295	1.6655	1.232	1.274	1.555	1.253	1.497667
5	1.865	1.4755	1.8895	1.8465	1.269	1.749	1.734	1.448	1.7475	1.515833
7	1.2065	1.6065	0.8325	1.3545	1.287	1.072	1.1535	1.5915	0.9865	1.454

Table 3. Level of calcium deposition of MG63 osteoblastic cell culture after 3 and 7 day of injectable pastes application

Day	IBX (concentration)			IHA-C (concentration)			IHA (concentration)			Control
	1%	0.50%	0.25%	1%	0.50%	0.25%	1%	0.50%	0.25%	
3	860	900	940	900	960	980	1.02	1010	1.04	230
7	1.340	1.380	1.440	1.340	1.360	1.460	1.600	1.540	1.460	660

The viability of the test group of HA based pastes (IHA and IHA-C) did not have toxic effect rather they increased cell proliferation and ALP secretion. The result in line with a report from Karaj *et al.*,[7] which found that HA is able to increase cells proliferation. However, the IHA 0.25% group was the lowest in producing ALP at fifth day of exposure, compared with IBX 0.25% and IHA-C 1%. These results probably because IHA paste only contained HA. Serre *et al*.,[8] reported that biomaterial which contained HA and collagen can increase protein matrix synthesis more than a biomaterial with HA only.

The addition of chitosan in HA as bone substitute material was expected to improve the biocompatibility of the biomaterial. Chitosan with degree of deacetylation (DDA) greater than 99% could improve bone regeneration process at a defect in the femurs of sheep.[9] Chitosan also had an positive effect on cartilage regeneration.[10–12] However, the result in this study showed the cells viability percentage of IHA-C group was lower than the IHA group. One argument to explain this finding is the DDA of the chitosan in IHA-C paste. The range of DDA is commonly 70–90% and the ability to induce cell proliferation increases with the rise of DDA.[5] Unlike the cells proliferation, the ALP secretion of IHA-C group is higher than IHA group. This result shows that alkaline phosphatase expression as an initial marker of bone regeneration process in in-vitro. The result of this study indicates that all injectable pastes increasing osteoblast cell differentiation, showed by the height of alkaline phosphatase expression. This was also reported in previous in-vitro studies in cell culture by Takamori *et al.,*[13] and Joss *et al*.[15]

Based on the data presented in this study, cell viability percentage of IBX group was the lowest compared with the other paste groups. This phenomenon probably because the IBX paste contain bovine xenograft which taken from bovine bones loosed some of their organic components and pathogens during the chemical extraction.[3,13] Besides that, the size of the particles can also influence the viability percentage. It is believed that smaller particle size can improve the properties of synthetic bone substitute, due to its higher surface area.[7] The size of bone xenograft in IBX is 60 mesh, and the Ha particle in IHA and IHA-C is 100 mesh. The ALP secretion research showed that the IBX paste groups produced more ALP than the other two pastes groups. This is probably because the bovine xenogaft still have a small amount of BMP which can stimulate cells differentiation.[14] The discrepancies of the results in the cell proliferation and ALP secretion also appropriated with the report of Joss *et al*.[15] which stated that when there is an increment in ALP production during the exposure of bovine extract on osteoblast cells, the amount of cell proliferation decreased.

In this study Von Kossa staining showed the increment of calcium deposition in human osteoblast cell culture exposed with IBX, IHA and IHA-C compared to control group. The highest calcium deposition was shown on 1% of IHA at day 7 followed by 1% IHA-C and 1% of IBX ($p > 0.05$). Similar finding reported by Carnes *et al.*[17] and Shen *et al.,*[18] showing that early calcium deposition in osteoblast cell culture reached at day 7. Therefore, this result proved that all form of injectable pastes have the ability to increase mineralization of osteoblast cell in bone regeneration process.

Biocompatibility and osteoconductive properties of IBX, IHA-C and IHA were evaluated in this study and resulted no toxic effect of these materials as indicated by cell viability that was assayed by MTT test. In addition, the secretion ALP as early biomarker of osteogenesis occured from day 3 and increase at day 7, whereas mineralization were detected at day 7 of culture.

This study showed the biological activities of osteoblast as its responses to the injectable pastes tested. After 24 hours, we have observed an increment in the percentage of cellular viability in the treatment group as compared to that of the control group. This result suggests the possibility of observing biocompatibility property of bone graft pastes 24 hours post treatment period. In addition, we have observed an ALP secretion in the osteblasts culture. This observation and in addition to the observed ALP 7 days turnover rate indicates a possible role of this molecule on calcium deposition.[8] In almost all of the experimental groups, the ALP were secreted in small amount in day 3. In this experiment, ALP secretion rate rose to its peak on day 5 and subsequently dropped in day 7 of culture and at this time calcium deposition was detected.

In conclusion, all bone graft pastes seem to be biocompatible as indicated by the MTT assay and have an osteoconductivity capability based on the ALP secretion and calcium deposition. Evaluation of the osteogenic property of pastes on Macaca's mandibular bone is currently ongoing. The results of this work is significant because of the potential usage of the injectable scaffold tested here, which can be considered as an alternative source of effective and affordable biomaterials for tissue engineering. The outcome

of this research could help to reduce our dependency on imported scaffolding biomaterials for an affordable bone reconstructive treatment.

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