Osteogenesis Effect of Human Adipose-derived Mesenchymal Stem Cell with Chitosan Scaffold in White Rat (*Rattus norvegicus*) Bone Defect on Serum Alkaline Phosphatase

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Abstract

Bone defect is one of the challenges for dentists in the process of healing bone tissue. Bone defect can occur in alveolar bone with the etiology of microorganisms and cyst expansion. In addition, cases of bone defects in alveolar bone are also often found in cases with treatment of apex resection and hemisection. Autologous bone graft is a clinical gold standard in the treatment of bone defect. However, the use of bone graft has a limited number of growth factors produced. Tissue engineering is the latest method in terms of bone regeneration. Tissue engineering has three main components; stem cell, growth factor, and scaffold. Stem cells will increase osteoblastogenesis and chitosan scaffold will immobilize alkaline phosphatase (ALP) so that serum ALP levels decrease and bone regeneration and mineralization processes become faster.

The aim of this study is to analyze osteogenesis effect of human adipose-derived mesenchymal stem cell (HADMSC) with chitosan scaffold (CS) in white rat bone defect on serum alkaline phosphatase (ALP) levels.

This research was a in vivo laboratory experimental study. Bone defects are planted with chitosan scaffold (CS) and a combination of human adipose-derived mesenchymal stem cells (HADMSC) with chitosan scaffold. Measurement of ALP levels was carried out by the International Federation of Clinical Chemistry (IFCC) method using an analyzer on the 1st, 3rd, 7th and 14th days. Research data were analyzed using multivariate analysis of variance (MANOVA) and Bonferroni tests.

The results of the data analysis showed that there were significant differences in ALP levels with CS planting and the combination of HADMSC and CS. Osteogenesis effect of human adipose-derived mesenchymal stem cell (HADMSC) with chitosan scaffold (CS) in white rat bone defect reduces serum alkaline phosphatase (ALP) levels on the 3rd and 14th days.

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Introduction

Bone is the second most transplanted tissue in the body with approximately 3.5 million bone graft procedures performed each year. There are many cases in which bone grafts are needed in large quantity such as for reconstruction of large bone defects caused by trauma, tumors, infections, and congenital defects.[1] Tissue engineering is one of some solution for bone defect treatment. Tissue engineering consists of cells, scaffolds, and growth factors that are used to restore bodily functions. Treatment of tissue engineering in

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bone defects in the last 3 decades is autogenous bone graft and is considered to be a clinical gold standard. However, autogenous sources used tend to be limited. Clinicians have switched to substitution of allogenic bones such as demineralized bone matrix consisting of extracellular matrix proteins and minimum growth factors. However, the release of growth factors in the use of allogenic bone substitution is limited to long-term use. The current lack of methods shows the need to combine bone-forming cells.[2]

Mesenchymal stem cells (MSC) offer promising cell sources in scaffold-based tissue engineering because of their multipotent differentiation capacity and tissue regeneration ability.[3] MSC is commonly found in the bone marrow or often called bone marrow mesenchymal stem cells (BMMSC). However, considering the conditions for obtaining BMMSC such as invasive bone marrow aspiration which causes severe pain and trauma and the number of cells obtained is small, making BMMSC a poor source to obtain MSC.[4]

Human adipose-derived mesenchymal stem cells (HADMSC) were first documented in 2001 from lipoaspiration as a source of stem cells.[5] The advantages of HADMSC compared to BMMSC are that it can be stored in vitro for a longer time by doubling a stable population, greater proliferative capacity and lower aging rates than BMMSC. According to Chen et al. and Wu et al., osteogenic potential and BMMSC cell proliferation potential decrease with age, whereas osteogenic potential and proliferation potential of HADMSC are not significantly affected by age.[6,7] Chen et al. comparing the differentiation of osteogenic HADMSC and BMMSC between young groups (age 36.4 ± 11.8 years) and the elderly group (age 71 ± 3.6 years). Their results indicate that the level of HADMSC matrix mineralization from young patients is comparable to older patients, whereas BMMSC in older patients produces fewer mineral deposits and has lower osteogenic gene expression levels compared to young groups.[6]

The application of HADMSC in bone defect requires a scaffold so that the HADMSC network does not experience denaturation. Some of the scaffolds currently used are collagen, chitosan, gelatin, alginate, fibrinogen, and hyaluronic acid.[8] Each scaffold has advantages and disadvantages based on the targeted tissue. Chitosan scaffold (CS) is the material of choice in bone regeneration because CS has a structural similar to glycosaminoglycan.

Glycosaminoglycan is one of the main components connected with collagen fibers in extracellular matrix (ECM).[9]

The mechanism of bone repair and regeneration from HADMSC consists of 4 main pathways, namely signaling mothers against decapentaplegic (Smad), indian hedgehog (ihh), Wnt canonical, and mitogen-activated protein kinase (MAPK). These four pathways will stimulate the formation of osteochondroblast progenitors into committed pre-osteoblasts. When the pre-osteoblast committed phase there are alkaline phosphatase (ALP), collagen1a1 (Col1a1), and PTH-receptor1 (PTHR-1) which will help the process of maturation into osteoblasts. ALP levels will increase when the four pathways are stimulated by HADMSC so that bone repair and regeneration can run faster. In addition, CS can result in immobilization of ALP so that serum ALP levels decrease and increase bone mineralization. Therefore, serum ALP levels are the material that needs further investigation to measure the level of osteoblast formation.[2,10]

Materials and methods

Research Samples

The experimental animals used in this study were male rats (Rattus norvegicus) 3 months old wistar strain with an initial body weight of 200-230 gr as many as 36 rats with healthy conditions obtained from UD. Eternal Jaya Tiput. The white rat (Rattus norvegicus) is strung on a trial cage made of a plastic tub and given a cage cover made of fence wire. Rat cages are conditioned in clusters that are placed in a place with equal temperature and humidity between groups. Rats are kept and adapted for one week in a cage and given feed in the form of pellets and drink in ad libitum.

Research Methods

1. Sample preparation stage

Experimental animals are randomly selected and divided into 12 groups: The GR1 group is the negative control group as normal

bone which is drilled. ALP levels in blood serum were checked on day 1, The GR3 group is the negative control group as normal bone which is drilled. ALP levels in blood serum were checked on the 3rd day, The GR7 group was the negative control group as a normal bone which is drilled. ALP levels in the blood serum were checked on the 7th day, The GR14 group was the negative control group as a normal bone which is drilled. ALP levels in blood serum were checked on day 14. The GRS1 group is the positive control group with the administration of chitosan scaffold after bone drilling. ALP levels in blood serum were checked on day 1. The GRS3 group was the positive control group with the administration of chitosan scaffold after bone drilling. ALP levels in blood serum were checked on the 3rd day. The GRS7 group was the positive control group with the administration of chitosan scaffold after bone drilling. ALP levels in the blood serum were checked on the 7th day. The GRS14 group was the positive control group with the administration of chitosan scaffold after bone drilling. ALP levels in blood serum were checked on day 14. The treatment group GRP1 is the group with the administration of HADMSC on chitosan scaffold after bone drilling. ALP levels in blood serum were checked on day 1. The treatment group GRP3 is the group with the administration of HADMSC on chitosan scaffold after bone drilling. ALP levels in blood serum were checked on the day 3. The treatment group GRP7 is the group with the administration of HADMSC on chitosan scaffold after bone drilling. ALP levels in blood serum were checked on day 7. The treatment group GRPP14 is the group with the administration of HADMSC on chitosan scaffold after bone drilling. ALP levels in blood serum were checked on day 14.

2. Human Adipose-derived Mesenchymal Stem Cell Isolation and Characterization

Freshly taken lipoaspirate and washed with PBS 1% sterile until golden in color. The adipose tissue was then dissolved with a solution of 0.01% collagenase or made in PBS solution with a ratio of 1 ml of enzyme solution and 1 cm^3 of adipose tissue. This mixture then incubated at 37°C with intermittent agitation until it is completely mixed (usually 30 minutes). Infranatants were then carefully suctioned, transferred in 50 ml conical tubes and centrifuged at 706 x g for 8 minutes with PH 7.3. The supernatant was removed and produced a pellet SVF, resuspended to the media control of Dulbecco's Modified Eagle's Medium Low Glucose (DMEM-LG) supplemented with 10% serum bovine fetal (FBS), penicillin 500 IU and streptomycin 500lg (Mediatech, Manassas, VA, USA). Cells were then counted on T75 flasks that were not coated with a concentration of 1 x 10^6 cells. Then 20 mg lipoaspirate is sufficient tissue to produce adequate SVF (> 1 x 10^7 cells)[12]. Mononuclear cells were coated in expansion medium (MI) at a density of 10^5 cells / cm2 in tissue culture coated with 10 ng/ml fibronectin (Sigma, Deisenhofen, Germany). Media expansion consists of 58% Dulbecco's Modified Eagle's Low Glucose Medium (DMEM-LG, Cambrex, Apen, Germany) and 40% MCDB201 (Sigma), 2% fetal calf serum (FCS; StemCell Technologies, Vancouver, BC, Canada), equipped with 2mM L-glutamine, 100 U / ml Pena / Strep (Gibco, Enggestein, Germany), 1% insulin transferrin selenium, 1% linoleic acid bovine serum albumin, 10 nM dexamethasone, and 0.1 mM L-ascorbic acid-2 -phosphate (all from Sigma), platelet-derived growth factors, and epidermal growth factors (10 ng / mL each). When it reaches 80% confluence, cells are tested with 0.25% trypsin / 1 mM EDTA (Invitrogen, Karlsrube, Germany) and replaced at around 9,000 cells / cm^2, cells are expanded for 2 to 6 passages.[11]

Then, we identify HADMSC with the phenotypic mesenchymal stem cell (MSC) kit after the second passage. HADMSC on the expressions of CD73, CD90, and CD 105 has no expression also in expression of CD45, CD34, CD14, or CD11b, CD 79a, and HLA-DR. Cells were analyzed using Accurl C6 Flowcytometer (BD Biosciences, San Jose, CA, USA) which showed positive staining for CD90 (81.3%), CD105 (86.6%), and CD73 (99.9%) and negative coloring for CD14, CD20, CD34, and CD45. After that, these cells can be identified in the adipogenic, osteogenic or chondrogenic conditions available in the kit (Cyagen Biosciences Inc., Sunnyvale, CA, USA).

3. Chitosan Scaffold Toxicity Test for HADMSC

Toxicity test of chitosan scaffold on HADMSC cell culture was carried out with trypsination from one petri plate which covered 2.5 x 10^6 cells. Then resuspension into DMEM / F12 medium and centrifugation. Pellets were grown into culture with 96 wells (M96) each of 5 x 10^4 cells/wells then incubated for 24 hours at 37°C and 5% CO_2. When 80% of the growth is obtained, chitosan scaffold inserted into ½ parts of the well that have contained cells. DMEM / F12 medium was added as much as 100 microliters, then incubated again for 20 hours at 37°C and 5% CO2. After that, MTT was added 5 mg/mL (25 microliters / well), then incubated for 4 hours and watch under the inverted microscope. Scaffold and medium removed then added sDMSO 200 microliter/well. Each the well readed by Elisa Reader at a wavelength of 595 nm.

4. HADMSC seeding in Chitosan Scaffold

Cell suspension containing 5x10^5 cells in 100 µl medium was poured on the surface of each scaffold placed on unprocessed six-well non-tissue culture plates. Each construct was incubated at 37° C for 1 hour. After 1 hour, 4.5 ml of chondrogenic media are added to each well. Plates are placed on an oscillating shaker platform 60 rpm. Construction was maintained at 37° C with 5% CO2 in chondrogenic media for 72 hours, 10 days or 21 days. The medium is changed three times a week.

5. Transplantation of HADMSC and Chitosan Scaffold into Bone Defect

Anesthetic injection is performed using ketamine HCl 50 mg / Kg BB and xylazine HCl 10 mg / Kg intramuscularly in the femoral caudal extremity. Anesthetized rats are shaved in the ventral hair with a dorsal recumbency position. Antiseptic application is applied using cotton containing calium iodide and applied to shaved rats skin. The incision is carried out using a blade on the superficial skin of the maxillary bone, approximately 1 cm under the eye. After the incision is successful and the maxillary bone is visible, the maxillary bone is drilled using a slow-speed hand piece with 1500 rpm speed to 3mm lateral and 2mm profundus. During drilling, sterile saline solution is given.

Transplantation of chitosan scaffold containing HADMSC in rats were carried out after drilling completed. Positive control groups were drilled and planted with scaffold whereas, the negative control group were only drilled and administrated of sterile saline solution. Bone closure is done by suturing on 2 layers of tissue. After suturing in the first layer, intraperitoneal enrofloxacin was injected. The sutured skin is given an antiseptic on the surface of the suture and is given a sterile gauze and covered with surgical tape plaster and bandaging with wound tape.

6. ALP serum study

All groups of experimental animals were given uniform standard feed during adaptation until the end of the treatment. On day 1, 3, 7 and 14, experimental animals were sacrificed to take the maxilla. Control group and treated group rats were anesthetized using ketamine HCl at a dose of 50 mg/Kg . After the rats did not show a painful response, the rat was laid down on top of the dissecting tray. Each leg is fixed with a needle. All skin and hair are moistened. The opening of the abdominal space is done by means of the abdominal muscles (abdominal wall) being cut in linea alba starting from the tip of the breastbone (process xiphoideus) to the pubic pectin ossis. After opening the abdominal wall, the chest cavity is opened, with the last rib cut to the front toward the sternal bone arch. Cutting is done on the right and left side until the heart is visible so that blood collection using a syringe through the right atrium of the heart can be done. Then, we centrifuge the blood to get blood serum. Blood ALP level were examined using IFCC method.

Statistical methods

This study was conducted to analyze ALP levels in the serum of mice planted with chitosan scaffold (CS). CS contains human adipose-derived mesenchymal stem cells (HADMSC) which stimulates bone regeneration. Data on ALP levels were tested for normality, homogeneity, and significance using multivariate analysis of variance (MANOVA).

Results

Measurements of alkaline phosphatase (ALP) levels in this study used the International Federation of Clinical Chemistry (IFCC) methods and the results were read with analyzer. The results of the mean and standard intersections of

ALP levels in 12 groups can be seen in table 1 until table 4.

Sample	Day One		
	GR (without chitosan scaffold and HADMSCs)	GRS (with chitosan scaffold but without HADMSCs)	GRP (with chitosan scaffold and HADMSCs)
Mean ± SD	327.3333 ± 51.73329	339 ± 73.30075	229.6667 ± 37.07200

Table 1. Mean Level and standard deviation of alkaline phosphatase (ALP) levels Day 1.

Sample	Day Three		
	GR (without chitosan scaffold and HADMSCs)	GRS (with chitosan scaffold but without HADMSCs)	GRP (with chitosan scaffold and HADMSCs)
Mean ± SD	568.3333 ± 33.29164	425.3333 ± 30.08876	326.6667 ± 59.65177

Table 2. Mean Level and standard deviation of alkaline phosphatase (ALP) levels Day 3.

Sample	Day Seven		
	GR (without chitosan scaffold and HADMSCs)	GRS (with chitosan scaffold but without HADMSCs)	GRP (with chitosan scaffold and HADMSCs)
Mean ± SD	476.6667 ± 61.10101	339.3333 ± 65.12552	382.6667 ± 28.02380

Table 3. Mean Level and standard deviation of alkaline phosphatase (ALP) levels Day 7.

Sample	Day Fourteen		
	GR (without chitosan scaffold and HADMSCs)	GRS (with chitosan scaffold but without HADMSCs)	GRP (with chitosan scaffold and HADMSCs)
Mean ± SD	453.0000 ± 10.81665	264.0000 ± 10.00000	313.3333 ± 14.74223

Table 4. Mean Level and standard deviation of alkaline phosphatase (ALP) levels Day 14. GR1,3,7,14: ALP levels on day 1, day 3, day 7, and day 14 without planting chitosan scaffold and human adipose-derived mesenchymal stem cells (controls), GRS1,3,7,14: ALP level on day 1, day 3, day 7, day 14 by planting chitosan scaffold without human adipose-derived mesenchymal stem cells, GRP1,3,7,14: ALP level on day 1, day 3, day 7, and day 14 by planting chitosan scaffold and human adipose-derived mesenchymal stem cells.

Based on table 1 until table 4 the highest mean value of ALP in the GR3 group was 568.33333 U/L and the lowest in the GRP1 group was 229.6667 U/L. To make it easier to see the comparison of the mean values of measurement of ALP levels in each group, the results of the study can be illustrated in the bar graph (figure 1).

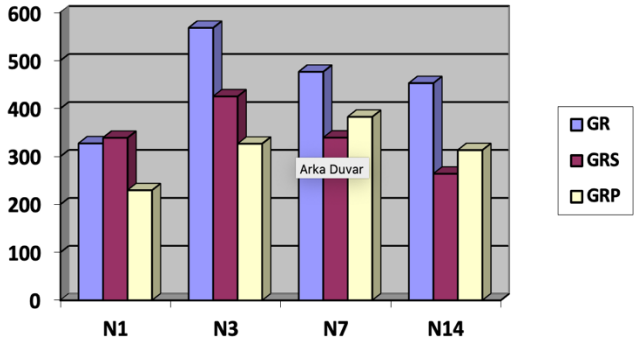

Figure 1. Graph comparison of the average value of ALP levels for each group.

Data from the research results were analyzed by the Shapiro-Wilk test to find out the results of the ALP test data for each study group with normal distribution. The research data is normally distributed if the probability value is greater than 0.05 (p> 0.05). The results of the Shapiro-Wilk test in this study found that all sample groups had p> 0.05 so it could be concluded that the research data group was normally distributed.

The data were then tested using the Levene test to determine the homogeneity of the ALP level research data. The research data shows homogeneity if the probability value is greater than 0.05 (p> 0.05). The results of the Levene test data show a probability of 0.292 in ALP 1, 0.300 in ALP 3, 0.462 in ALP 7, and 0.608 in ALP 14 so that it can be concluded that the research data is homogeneous.

No	Effect		Value	F	Hypothesis DF	Error DF	P value
1	Treatment	Pillai's Trace	1,776	7,928[a]	8,000	8,000	0.004
2		Wilks' Lambda	0,001	19,416[b]	8,000	6,000	0.001
3		Hotelling's Trace	159,944	39,986[c]	8,000	6,000	0.001
4		Roy's Largest Root	156,349	156,349[d]	4,000	4,000	0.000

Table 5. Multivariate analysis of variance (MANOVA) results. The different superscript letters are statistically significant (MANOVA, P < 0.05).

The requirement to conduct a multivariate analysis of variance (MANOVA) test is that the research data is normally distributed and homogeneous. Based on the results of the Shapiro-Wilk test and the Levene test, the research data were normally distributed and homogeneous, so the research data qualified for the MANOVA test. The MANOVA test was

conducted to determine the existence of significant differences in all treatment groups and the overall control group. The research data shows a significant difference if the probability value is smaller than 0.05 ($p < 0.05$). The results of the MANOVA test can be seen in table 5.

Based on table 5, the results of the research data test using MANOVA show probability values $p < 0.005$ so that it can be concluded that there are significant differences in all sample groups. It can be interpreted that ALP levels in the group without planting chitosan scaffold and human adipose-derived mesenchymal stem cells, planting chitosan scaffold without human adipose-derived mesenchymal stem cells, and planting human adipose-derived mesenchymal stem cells with chitosan scaffold there were significant differences whole. In addition, the MANOVA test displays Tests of Between-Subjects Effects as in table 6.

No	Source		Mean Square	P Value
1	Treatment	ALP 1	1224.333	0.693
2		ALP 3	44293.444[a]	0.001
3		ALP 7	14787.111	0.051
4		ALP 14	28830.778[b]	0.000

Table 6. Tests of Between-subjects Effects. Mean of each group based on ALP day one, three, seven, and fourteen. The different superscript letters are statistically significant (Tukey HSD, $P < 0.05$).

	ALP 1		ALP 3		ALP 7		ALP 14	
G	GRS1	GRP1	GRS3	GRP3	GRS7	GRP7	GRS14	GRP14
GR1	1.000	1.000						
GRS1		1.000						
GR3			0.020[a]	0.001[b]				
GRS3				0.093				
GR7					0.062	0.231		
GRS7						1.000		
GR14							0.000[c]	0.000[d]
GRS14								0.007[e]

Table 7. Bonferroni Tests. The different superscript letters are statistically significant (Bonferroni, $P < 0.05$).

The results of the MANOVA data analysis test showed that there were significant differences in some groups of samples, so the Bonferroni test was needed to determine the significant differences between the sample groups. The results of the Bonferroni test data show a significant difference if the probability value is equal to 0.05 ($p < 0.05$). The Bonferroni test results can be seen in table 7.

The results of the research data using the Bonferroni test showed significant differences in the N3 GR group with the N3 GRS and N3 GRP groups. Significant differences were again found in the N14 GR group with N14 GRS and N14 GRP and between the N14 GRS and N14 GRP groups. The results of the Bonferroni test data there are no significant differences between groups, namely in groups other than experiencing significant differences.

Discussion

Tissue engineering is a cutting-edge strategy that aims to restore the function of a network that has a defect. Tissue engineering has three components, namely growth factors, biomaterial scaffolds, and cells. Absolute bone regeneration process requires osteoconductive, osteoinductive scaffold and provides a microenvironment suitable for proliferation, differentiation and tissue formation.[13,14]

The use of Human adipose-derived mesenchymal stem cells (HADMSC) in this study is based on the successful use of HADMSC in clinical trials conducted from 2004 to 2014. Liao and Chen, proves that HADMSC successfully triggers bone regeneration in the craniomaxillofacial region and without causing an immune rejection response the host.[5]

The application of HADMSC to bone tissue requires a scaffold so that the HADMSC network does not experience denaturation. Chitosan scaffold (CS) was chosen because chitosan scaffold has a structure similar to glycosaminoglycans.[9] Glycosaminoglycans are one of the main components connected with collagen fibers in the extracellular matrix (ECM). CS has a hydrophilic surface that can increase proliferation and attachment of osteoblasts so that the combination of HADMSC and chitosan scaffold can lead to pathways of osteoblastogenesis through the pathway of signaling mothers against decapentaplegic (Smad), Wnt canonical, indian hedgehog (ihh), and mitogen-activated protein kinase (MAPK).[2]

The Smad pathway begins when bone morphogenetic proteins (BMPs) initiate cascade signals through the ligand that bind to the heteromeric complex of serine/threonin kinase

types I and II on the cell surface. The bond activates phosphorylate transcription factor receptors, namely signaling mothers against decapentaplegic (Smad) protein 1, 5 and 8. Smads forms a heterodimer complex with Smad 4 and target gene expression that promotes osteogenic differentiation. In addition, BMPs also increase the transcription of core-binding factor-1/runt-related transcription factor 2 (Cbfa1/Runx2) which is a regulatory molecule for differentiation along the osteoblast lineage.[2]

The Wnt canonical path begins with a bond between frizzled (Fz) and low-density lipoprotein (LDL) -receptor-related protein 5/6 (LRP5/6). The Fz-LRP5/6 complex pathway and Wnt canonical signaling produce β-catenin stabilization and translocation to the nucleus so that it will bind to the T cell factor/lymphoid enhancer factor (TCF)/Lef transcription factor. The β-catenin-TCF/Lef complex activates transcription of various Wnt-responsive genes, including genes involved in proliferation, osteoblast differentiation and osteogenesis.[2,15]

The Indian hedgehog (ihh) pathway is stimulated by binding of the hedgehog (Hh) protein to the patched (Ptch) receptor. When there is no interaction from Hh to Ptch, Ptch suppresses the signal transducer activity of 7 smoothened transmembrane proteins (Smo). Conversely, binding of Hh to Ptch prevents the suppressive effect of Ptch allowing activation of Smo thereby activating intracytoplasmic Gli protein (Gli in mammals and Ci in Drosophila). 2 One of the Gli proteins that has a strong activator ability is Gli1 (GliA). GliA can activate gene targets through interaction with specific DNA binding elements.[2,10]

The MAPK pathway can phosphorylate Runx2 and distal-less homeobox 5 (Dlx5) for activation, implying that MAPK is an obligatory transducer for bone healing. Previous research has shown transient upregulation of dual leusin zipper kinase (DLK) that functions on the MAPK pathway. Therefore, the pathway can be induced during bone healing even though the exact mechanism and molecular factors involved are unknown. In addition, the MAPK pathway can be activated by pro-inflammatory elements such as tumor necrosis factor α (TNF-α). The role of inflammation in improving bone defect has been largely explained with a special focus on TNF-α. TNF-α also activates the MAPK pathway during the inflammatory stage to induce MSC proliferation and inhibits differentiation of MSC. These four pathways will stimulate the formation of osteochondroblast progenitors into committed pre-osteoblasts. When the pre-osteoblast committed phase there are alkaline phosphatase (ALP), collagen1a1 (Col1a1), and PTH-receptor1 (PTHR-1) which will help the process of maturation into osteoblasts. ALP levels will increase when the four pathways are stimulated by HADMSC so that bone repair and regeneration can run faster.[2,10]

Research on ALP levels has been carried out in white rats (Rattus norvegicus) with groups without chitosan scaffold (CS) and human adipose-derived mesenchymal stem cells (HADMSC) (GR), CS planting groups without HADMSC (GRS), and CS planting groups HADMSC (GRP). Normal ALP levels in Rattus norvegicus are 30-130 U/L. 16 Based on table 1 all groups have above average values. This shows that making bone defect increases ALP levels. High ALP levels indicate high differentiation of osteoblasts.[2,10]

The results of the multivariate analysis of variance (MANOVA) data analysis (table 2) show that ALP levels in the GR, GRS and GRP groups have significant differences overall. In addition, the MANOVA test also displays Tests of Between-Subjects Effects (table 3). The test showed that in the ALP 3 and ALP 14 groups showed significant differences between the treatment groups while in the ALP 1 and 7 groups did not show a significant difference.

The results of data analysis using the Bonferroni test (table 4) show that in the N1 GR group there were no significant differences in the N1 GRS and N1 GRP groups. This is possible because of the inflammatory effects that activate TNF-α. TNF-α activates the mitogen-actived protein kinase (MAPK) pathway thereby inducing proliferation of HADMSC and inhibiting the differentiation of HADMSC into osteoblasts. Differentiation barriers cause the formation of ALP on day 1 low.[2,10]

The results of data analysis using the Bonferroni test (table 4) showed that the GR3 group had a significant difference to the GRS3 and GRP3 groups. This happened because the ALP levels in the GR3 were likely to increase due to proliferation barriers on the MAPK pathway. These obstacles result in differentiation of MSC proliferation on the first and second day.[2,10] However, GRS3 and GRP3 do not have

significant differences. This happens because of the effect of immobilization of CS on ALP.[16,17]

The results of data analysis using the Bonferroni test (table 4) showed that the GR7 group did not have a significant difference to the GRS7 and GRP7 groups. The GRS7 also does not show a significant difference in the GR7. This happened because the ALP levels in the GR7 decreased while GRP7 levels increased. Decrease in ALP GR7 levels is due to the process of bone homeostasis to adjust the ALP levels produced with the amount of phosphate produced.[18] In addition, formation of osteoblasts that are high on day 3 will stimulate dickkopfs 1 (DKK1). DKK 1 is a Wnt antagonist that binds Lrp5/6 so that the inhibition of osteochondroblast progenitor formation into committed pre-osteoblast occurs. These obstacles result in a decrease in ALP levels.[10]

The results of data analysis using the Bonferroni test (table 4) show that the GR13 group has a significant difference to the GRS14 and GRP14 groups. This is because of the influence of chitosan scaffold in ALP immobilization. Chitosan scaffold can immobilize ALP through biomolecule physical adsorption method in CS, chemical adsorption of biomolecules to CS, and covalent bonds so that serum ALP decreases. Biomolecular physical adsorption occurs because of ion and hydrogen bonds, hydrophobic interactions, and van der Waals forces. Chemical adsorption of biomolecules can be obtained by binding to covalent biomolecules with functional groups of agents. The covalent biomolecule bonds occur by binding biomolecules through unique attachment directly to chitosan scaffold.[17]

In addition, the GRS14 and GRP14 groups also have significant differences. This happened because the GRP14 group had HADMSC that experienced entrapment in CS. CS that was degraded caused ALP release which experienced entrapment in CS.[17,19,20]

Conclusions

Osteogenesis effect of human adipose-derived mesenchymal stem cell (HADMSC) with chitosan scaffold (CS) in white rat bone defect reduces serum alkaline phosphatase (ALP) levels on the 3rd and 14th days.

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Declaration of Interest

The authors declare no conflicts of interest.

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