THE EFFECT OF HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELL (HADMSC) WITH CHITOSAN SCAFFOLD ON BONE DEFECT WHITE RATS (Rattus norvegicus) ON SERUM ALKALINE PHOSPHATASE (ALP) LEVELS

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

Keywords: Chitosan scaffold; HADMSC; ALP level; ALP immobilization

INTRODUCTION

Bone defect is the loss of bone tissue volume in certain areas of the body (Smrke et al., 2013). Defective bone areas generally experience vascularity disorders (Smrke et al., 2013). Bone defects can occur in the alveolar bone with a microorganism etiology and cyst expansion (Penumatsa et al., 2013). Cases of bone defects in the alveolar bone are also frequently found in cases with apex resection and hemisection treatment. In addition, periapical tissue damage that causes cysts to form will result in cortical expansion and root resorption, causing bone defects (Kadam et al., 2014)

Tissue engineering is one solution for the treatment of bone defects. Tissue engineering consists of cells, scaffolds, and growth factors that are used to restore body functions. Tissue engineering treatment for bone defects in the last 3 decades is autogenous bone graft and is considered a clinical gold standard. The release of growth factors in the use of allogenic bone substitutes is limited to long-term use. The shortcomings of current methods indicate the need to incorporate bone-forming cells (Grottkau & Lin, 2013).

Mesenchymal stem cells (MSCs) are emerging as a promising source of cells in scaffold-based tissue engineering due to their multipotent differentiation capacity and tissue regeneration ability (Zang et al., 2017). Human adipose-derived mesenchymal stem cells (HADMSC) were first documented in 2001 from lipoaspiration as a source of stem cells (Liao & Chen, 2014). The advantages of HADMSCs over BMMSCs are that they can be stored in vitro for a longer time with stable population doubling, greater proliferative capacity and lower aging rates than BMMSCs.

The application of HADMSC to bone defects requires a scaffold so that the HADMSC tissue does not experience denaturation. Some of the scaffolds currently used are collagen, chitosan, gelatin, alginate, fibrinogen, and hyaluronic acid (Chang et al., 2017). The mechanism of bone repair and regeneration of HADMSC consists of 4 main pathways, namely signaling mothers against decapentaplegic (Smad), indian hedgehog (ihh), canonical Wnt, and mitogen-activated protein kinase (MAPK) (Deschaseaux et al., 2009)(Grottkau & Lin, 2013). These four pathways will stimulate the formation of osteochondroblast progenitors to become committed pre-osteoblasts. During the committed pre-osteoblast phase, there are alkaline phosphatase (ALP), collagen1a1 (Col1a1), and PTH-receptor1 (PTHR-1) which will help the maturation process into osteoblasts.

METHOD

The type of research used is in vivo laboratory experimental research. The research design used was post test only control group designs. Experimental animals used in this study were male white rats (Rattus norvegicus) wistar strain aged 3 months with an initial body weight of 200-230 g as many as 36 tails.

The sample size in this study (each group) was calculated using the Federer formula (Federer, 1974; Yuniati, 2011), as follows:

$(n-1)(t-1) \geq 15$

$(12-1)(n-1) \geq 15$

$n-1 \geq 15/11$

$n \geq 2,3$

$n \geq 3$

Description: n = number of samples for each treatment group

t = number of treatment groups

$(t-1)(n-1) \geq 15$

Based on the Federer formula listed above, 3 samples were used in each experimental group. This study used 36 rats which were divided into 3 main groups; negative control, positive control, and treatment.

RESULTS

Measurement of alkaline phosphatase (ALP) levels in this study used the International Federation of Clinical Chemistry (IFCC) method and the results were read using an analyzer. The results of the mean value and standard deviation of the ALP level test in 12 groups can be seen in the table.

Table 1. Average and standard deviation of alkaline phosphatase (ALP) levels

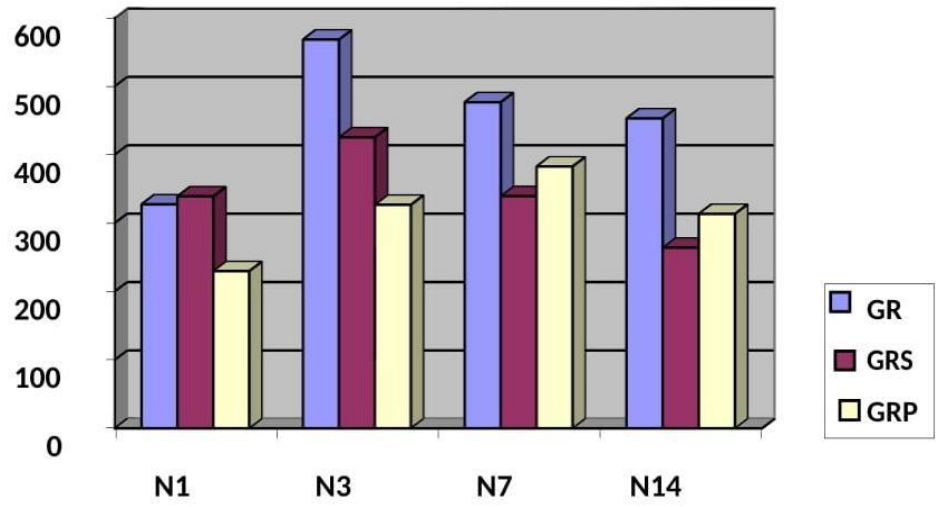
No	Group	n	Average ALP Levels (U/L)	*Std. Deviation* (SD)
1	N1 GR	3	327,3333	51,73329
2	N1 GRS	3	339,0000	73,30075
3	N1 GRP	3	229,6667	37,07200
4	N3 GR	3	568,3333	33,29164
5	N3 GRS	3	425,3333	30,08876
6	N3 GRP	3	326,6667	59,65177
7	N7 GR	3	476,6667	61,10101
8	N7 GRS	3	339,3333	65,12552
9	N7 GRP	3	382,6667	28,02380
10	N14 GR	3	453,0000	10,81665
11	N14 GRS	3	264,0000	10,00000
12	N14 GRP	3	313,3333	14,74223

Description :
n : Number of samples
SD : Standard deviation
N1 GR : ALP levels on day 1 without chitosan scaffold implantation and human adipose-derived mesenchymal stem cells (control).
N1 GRS : ALP levels on day 1 by planting chitosan scaffold without human adipose-derived mesenchymal stem cells.
N1 GRP : ALP levels on day 1 by implanting chitosan scaffold and human adipose-derived mesenchymal stem cells.
N3 GR : ALP levels on day 3 without chitosan scaffold implantation and human adipose-derived mesenchymal stem cells (control).
N3 GRS : ALP levels on day 3 by planting chitosan scaffold without human adipose-derived mesenchymal stem cells.

N3 GRP : ALP levels on day 3 by implanting chitosan scaffold and human adipose-derived mesenchymal stem cells.
N7 GR : ALP levels on day 7 without chitosan scaffold implantation and human adipose-derived mesenchymal stem cells (control).
N7 GRS : ALP levels on day 7 with chitosan scaffold implantation without human adipose-derived mesenchymal stem cells.
N7 GRP : ALP levels on day 7 by implanting chitosan scaffold and human adipose-derived mesenchymal stem cells.
N14 GR : ALP levels on day 14 without chitosan scaffold implantation and human adipose-derived mesenchymal stem cells (control).
N14 GRS : ALP levels on day 14 by planting chitosan scaffold without human adipose-derived mesenchymal stem cells.
N14 GRP : ALP levels on day 14 by implanting chitosan scaffold and human adipose-derived mesenchymal stem cells.

Based on table 1 the highest mean value of ALP levels in the N3 GR group is 568.3333 U/L and the lowest in the N1 GRP group is 229.6667 U/L. To make it easier to see the comparison of the average value of the measurement results of ALP levels in each group, the results of the study can be depicted in a bar graph.

Figure 1. Comparison graph of the average ALP levels of each group



The results of the study were analyzed using the Shapiro-Wilk test to find out the data from the ALP level test results for each study group were normally distributed. Research data is normally distributed if it has a probability value greater than 0.05 (p > 0.05). The results of the Shapiro-Wilk test in this study showed that the entire sample group had p> 0.05 so it can be concluded that the research data group was normally distributed.

The data was then tested using Levene's test to determine the homogeneity of the research data on ALP levels. The research data is homogeneous if the probability value is greater than 0.05 (p > 0.05). The results of the Levene test data showed a probability of 0.292 for ALP 1, 0.300 for ALP 3, 0.462 for ALP 7, and 0.608 for ALP 14, so it can be concluded that the research data is homogeneous.

The MANOVA test was conducted to determine whether there were significant differences in the entire treatment group and the control group as a whole. The research data shows a significant difference if the probability value is less than 0.05 (p < 0.05). MANOVA test results can be seen in table 2.

Table 2. Multivariate analysis of variance (MANOVA) test results

No	*Effect*		*Value*	F	*Hypothesis* DF	*Error* DF	Sig.
1	Treatment	Pillai's Trace	1,776	7,928	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	8,000	6,000	0,001
4		Roy's Largest Root	156,349	156,349[c]	4,000	4,000	0,000

Description :
DF : Free degrees
F : F count
Sig. : Probability

Based on the table, the results of the research data test using MANOVA show a probability value of $p < 0.005$ so it can be concluded that there are significant differences in all sample groups. This means that ALP levels in the group without chitosan scaffold and human adipose-derived mesenchymal stem cells implantation, chitosan scaffold implantation without human adipose-derived mesenchymal stem cells, and human adipose-derived mesenchymal stem cells implantation with chitosan scaffold were significantly different. whole. In addition, the MANOVA test displays Tests of Between-Subjects Effects as shown in table 3.

Table 3. Tests of Between-subjects Effects

No	*Source*		*Type* III *Sum of Squares*	DF	*Mean Square*	F	Sig.
1	*Treatment*	ALP 1	2448,667	2	1224,333	0,390	0,693
2		ALP 3	88586,889	2	44293,444	23,848	0,001
3		ALP 7	29574,222	2	14787,111	5,064	0,051
4		ALP 14	57661,556	2	28830,778	199,138	0,000

Description :
DF : Free degrees
F : F count
Sig. : Probability

The results of the Bonferroni test data showed a significant difference if the probability value was equal to 0.05 ($p < 0.05$). The results of the Bonferroni test can be seen in table 4.

Table 4. Bonferroni . Test

	ALP 1		ALP 3		ALP 7		ALP 14	
K	N1 GRS	N1 GRP	N3 GRS	N3 GRP	N7 GRS	N7 GRP	N14 GRS	N14 GRP
N1 GR	1,000	1,000						
N1 GRS		1,000						
N3 GR			*0,020	*0,001				
N3 GRS				0,093				
N7 GR					0,062	0,231		
N7 GRS						1,000		
N14 GR							*0,000	*0,000
N14 GRS								*0,007

Description :
k : Group
* : Significant differences

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 with N14 GRS and N14 GRPgroups as well as between the N14 GRS and N1GRP groups.

DISCUSSION

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

The results of data analysis using the Bonferroni test (table 4) showed that in the N1 GR group there was an insignificant difference between the N1 GRS and N1 GRP groups. This may be due to the influence of inflammation that activates TNF-. TNF-α activates the mitogen-activated protein kinase (MAPK) pathway, thereby inducing HADMSC proliferation and inhibiting HADMSC differentiation into osteoblasts. Barriers to differentiation caused low ALP formation on day 1 (Deschaseaux et al., 2009)(Grottkau & Lin, 2013).

The results of data analysis using the Bonferroni test (table 4) showed that the N3 GR group had a significant difference to the N3 GRS

and N3 GRP groups. This is because ALP levels in N3 GR may increase due to proliferation inhibition in the MAPK pathway. However, N3 GRS and N3 GRP did not have a significant difference. This happens because of theimmobilization effect of CS on ALP (Thirumavalavan & Lee, 2015).

The results of data analysis using the Bonferroni test (table 5.4) showed that the N7 GR group did not have a significant difference to the N7 GRS and N7 GRP groups. The N7 GRS also did not show a significant difference to the N7 GRP. This happened because ALP levels in N7 GR decreased while ALP levels in N7 GRP increased. The decrease in ALP N7 GR levels is due to the process of bone homeostasis to adjust the levels of ALP produced with the amount of phosphate produced (Penido & Alon, 2012). In addition, high osteoblast formation on day 3 will stimulate dickkopfs 1 (DKK1). DKK 1 is a Wnt antagonist that binds Lrp5/6 so that inhibition of the formation of osteochondroblast progenitors to become committed pre-osteoblasts occurs. This inhibition results in a decrease in ALP levels (Deschaseaux et al., 2009).

The results of data analysis using the Bonferroni test (table 4) showed that the N14 GR group had a significant difference to the N14 GRS and N14 GRP groups. This is because of the effect of chitosan scaffold in ALP immobilization. Chitosan scaffold can immobilize ALP through physical adsorption of biomolecules on CS, chemical adsorption of biomolecules on CS, and covalent bonds so that serum ALP decreases. Physical adsorption of biomolecules occurs due to ionic and hydrogen bonds, hydrophobic interactions, and van der Waals forces. Chemical adsorption of the biomolecule can be obtained by covalent binding of the biomolecule with the functional group of the agent. Covalent bonding of biomolecules occurs by binding of biomolecules through unique attachments directly to the chitosan scaffold (Thirumavalavan & Lee, 2015). In addition, the N14 GRS and N14 GRP groups also had significant differences. This happened because the N14 GRP group had HADMSCs that experienced entrapment in CS. The degraded CS causes the release of ALP which undergoes entrapment in the CS (Dash et al.,2011) (Thirumavalavan & Lee, 2015).

BIBLIOGRAPHY

Chang, B., Ahuja, N., Ma, C., & Liu, X. (2017). Injectable scaffolds: Preparation and application in dental and craniofacial regeneration. *Materials Science and Engineering: R: Reports*, *111*, 1–26.

Dash, M., Chiellini, F., Ottenbrite, R., & Chiellini, E. (2011). Chitosan—A versatile semi-synthetic polymer in biomedical applications. *Progress in Polymer Science*, *36*(8), 981–1014.

Deschaseaux, F., Sensébe, L., & Heymann, D. (2009). Mechanisms of bone repair and regeneration. *Trends in Molecular Medicine*, *15*(9), 29–417.

Grottkau, B., & Lin, Y. (2013). Osteogenesis of adipose-derived stem cells. *Bone Research*, *1*(2), 133.

Kadam, N., De Ataide, I., Raghava, P., Fernandes, M., & Hede, R. (2014). Management of large radicular cyst by conservative surgical approach: a case report. *Journal of Clinical and Diagnostic Research*, *8*(2), 239.

Liao, H., & Chen, C. (2014). Osteogenic potential: Comparison between bone marrow and adipose-derived mesenchymal stem cells. *World Journal of Stem Cells*, *6*(3), 288.

Penido, M., & Alon, U. (2012). Phosphate homeostasis and its role in bone health. *Pediatric Nephrology*, *27*(11), 48–2039.

Penumatsa, N., Nallanchakrava, S., Muppa, R., Dandempally, A., & Panthula, P. (2013). Conservative approach in the management of radicular cyst in a child: case report. *Case Reports in Dentistry*.

Smrke, D., Rožman, P., Veselko, M., & Gubina, B. (2013). Treatment of Bone Defects— Allogenic Platelet Gel and Autologous Bone Technique. *In Regenerative Medicine and Tissue Engineering*.

Thirumavalavan, M., & Lee, J. (2015). A Short Review on Chitosan Membrane for Biomolecules Immobilization. *J Mol Genet Med*, *9*(178), 862–1747.

Zang, S., Zhu, L., Luo, K., Mu, R., Chen, F., Wei, X., Yan, X., Han, B., Shi, X., Wang, Q., & Jin, L. (2017). Chitosan composite scaffol