LEVELS OF WISTAR CALCIUM SERUM (Rattus Norvegicus) IN HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS (hADMSCs) AND CHITOSAN SCAFFOLD BY OSTEOINDUCTION EXAMINATION

Destri Imania[1], Latief Mooduto[2], Dian Agustin[3]

[1]Faculty of Dentistry, Airlangga University
[2]Department of Conservative Dentistry, Airlangga University
[3]Department Conservative Dentistry, Airlangga University
*Corresponding author: destriimania@gmail.com

ABSTRACT

Bone tissue reconstruction with extensive damage is one of the challenges for dentists because its healing process of bone tissue. Bone graft is the gold standard for bone repair. However, the use of bone graft has a limited amount of tissue produced. Tissue engineering is the latest method in terms of bone regeneration. Tissue engineering has three main components, first is stem cells that have self renewal ability and multineage differentiation, second is bioreactor / growth factor, and then scaffold. The combination of hADMSC and chitosan scaffold, is expected to trigger osteoinduction shown by osteogenic markers such as calcium levels. Purpose to prove osteoinduction in a combination of Human Adiposed Derived Mesenchymal Stem Cell (hADMSC) and chitosan scaffold using blood serum calcium levels. Methods: This study uses 12 treatment groups with each group having 4 samples. Groups 1 to 4 were the negative control group at 1st,3rd,7th, and 14th days which maxillary bone drilling only. While groups 5 to 8 were the positive control group at 1st,3rd,7th, and 14th days which were given chitosan scaffold. Groups 9 to 12 were treatment group at 1st,3rd,7th, and 14th days which were given hADMSC and chitosan scaffold. Blood collection is carried out in each group to check serum calcium levels. Result there were differences in calcium levels in blood serum in each group. Conclusion the application of hADMSC and chitosan scaffold caused a significant change in serum calcium levels on the 1st, 3rd, 7th and 14th days which meant that the combination of hADMSC and chitosan scaffold could trigger osteoinduction.

Keywords: Human Adiposed Derived Mesenchymal Stem Cell, chitosan scaffold, tissue engineering, calcium

INTRODUCTION

Reconstruction of extensive bone tissue requires bone graft. Autografts are the "Gold standard" used for bone tissue reconstruction to date. However, autograft has a drawback, namely the limited amount of tissue that can be produced (Miranda et al., 2011).

Tissue engineering is able to regenerate tissue so that the new tissue produced is the same as the original tissue, this is an advantage of tissue engineering compared to the natural healing process (repair) which can produce scar tissue, (Kao et al., 2009). Tissue Engineering has three main components, namely growth factors or bioreactors, scaffolds, and cells (O'brien, 2011; Westhrin et al., 2015).

Stem cells are one of the main components of tissue engineering because they have the ability to self-renewal and multilineage differentiation (Jonathan, 2018). Bone marrow mesenchymal stem cells (BMSCs) are a source of stem cells commonly used in tissue engineering (Egusa et al., 2012). These cells are able to differentiate into several cell types such as chondrocytes, osteoblasts, adipocytes, fibroblasts and other mesenchymal tissues (Miranda et al., 2011). The source of stem cells has just been discovered from fat tissue, so they are called adiposed-derived stem cells (ASCs) and if they are obtained from humans, they are called human adipose derived mesenchymal stem cells (hADMSCs) (Eberli, 2011).

The bone regeneration process requires a scaffold that is osteoconductive, osteoinductive while providing an appropriate microenvironment for proliferation, differentiation and tissue formation (O'brien, 2011). Some of the scaffolds currently used are collagen, chitosan, gelatin, alginate, fibrinogen, and hyaluronic acid (Chang et al., 2017). Chitosan is a semicrystalline polysaccharide that can be easily derived from partial deacetylation of the natural polymer, chitin (Croisier & Jerome, 2013). Glycosaminoglycan is one of the main components connected to collagen fibers in the extracellular matrix (ECM) (Thein-Han et al., 2008), so chitosan is suitable as a scaffold in tissue engineering.

Osterix (OSX also known as Sp7) is a zinc finger transcription factor that is expressed by osteoblasts and is required for bone formation (Jensen et al., 2010). OSX is a transcriptional regulator that functions in late bone formation (Hayrapetyan et al., 2015). This study was conducted to analyze the osteoinduction potential of the incorporation of hADMSCs in chitosan scaffold made from shrimp shells by evaluating serum calcium levels.

METHOD

This research is an in vivo experimental laboratory research by looking at the serum calcium levels of wistar rats (Rattus norvegicus) due to human Adipose-derived Mesenchymal Stem Cells (hADMSCs) and chitosan scaffold implanted in the maxillary bone of wistar rats. The experimental animals used in this study were

male wistar rats (Rattus norvegicus) of the wistar strain, aged 3 months with an initial body weight of 300-330gr as many as 48 tails.

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

Description : n = number of samples for each treatment group

t = number of treatment groups

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

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

$n-1 \geq 15/11$

$n \geq 2,3$

$n \geq 3$

Based on the Federer formula listed above, the samples used for each experimental group were at least 3 individuals. Therefore, this study used 48 rats which were divided into 3 groups. The first group is the negative control group. The second group is the positive control group, the third group is the treatment with each group using a range of days d-1, d-3, h-7 and d-14.

The MTT Assay hADMSCs and Chitosan Scaffold test were carried out at the Stem Cell Research and Development Center, Universitas Airlangga. Animal treatment, research trials, and seeding of hADMSCs and Chitosan Scaffolds were carried out at the Experimental Animal Cage, Faculty of Veterinary Medicine, Airlangga University. Serum calcium readings using the O-Cresolphthalein Complexion method were carried out at the Central Health Laboratory of Surabaya.

RESULTS

On the first day, serum calcium levels in the treatment group, namely hADMSCs and chitosan scaffold, had the lowest values compared to the negative control group and the positive control group (Table 1).

Table 1 Research Results of Serum Calcium Levels in Wistar Rats on hADMSCs and Chitosan Scaffold (day 1).

FIRST DAY SERUM CALCIUM LEVELS (N1) (mg/dl)		
NEGATIVE CONTROL	POSITIVE CONTROL	TREATMENT
10,22	11,9	9,49
10,28	12,1	9,56
10,04	11,4	9,41
0,29	12,8	9,45
$\bar{X}$10,2075	$\bar{X}$12,05	$\bar{X}$9,4775

While on the third day, serum calcium levels in the negative group and the treatment group had an average value that was not far apart. The mean serum calcium level in both the negative, positive control group and the treatment

group on the third day decreased slightly compared to the first day (Table 2).

Table 2 Research Results of Serum Calcium Levels in Wistar Rats on hADMSCs and Chitosan Scaffold (3rd day).

THIRD DAY SERUM CALCIUM (N3) (mg/dl)		
NEGATIVE CONTROL	POSITIVE CONTROL	TREATMENT
9,87	12,2	9,19
10,3	11,3	9,49
9,89	12,5	9,58
9,9	11,4	9,49
$\bar{X}$9,99	$\bar{X}$11,85	$\bar{X}$9,4375

On the seventh day the mean of the negative, positive control group and the treatment group increased compared to the third day. Serum calcium levels in the treatment group experienced the highest increase compared to the negative control group and the positive control group (Table 3).

Table 3 Research Results of Serum Calcium Levels in Wistar Rats on hADMSCs and Chitosan Scaffold (day 7).

SEVENTH DAY SERUM CALCIUM (N7) (mg/dl)		
NEGATIVE CONTROL	POSITIVE CONTROL	TREATMENT
9,71	11,2	11,6
9,71	12	10,8
9,41	12,6	12,8
9,53	12,4	11,4
$\bar{X}$9,59	$\bar{X}$12,05	$\bar{X}$11,65

On the fourteenth day, the mean serum calcium level of the negative, positive control group, and the treatment group experienced a slight increase compared to the seventh day (Table 4).

Table 4 Research Results of Serum Calcium Levels in Wistar Rats on hADMSCs and Chitosan Scaffold (day 14).

FOURTH DAY SERUM CALCIUM LEVELS (N14) (mg/dl)		
NEGATIVE CONTROL	POSITIVE CONTROL	TREATMENT
9,48	12,6	12
10,1	12,8	12,8
10,06	12,9	12,6
10,04	12,7	12,9
$\bar{X}$ 9,92	$\bar{X}$ 12, 75	$\bar{X}$ 12,575

Based on the table above, it is known that the serum calcium levels of hADMSCs and chitosan scaffold on the 1st, 3rd, 7th, 14th day increased from day 7 to day 14. The highest average calcium levels for negative control, positive control, and treatment occurred on day 14.

Data Analysis

Data on the value of serum calcium levels in wistar rats on hADMSCs and chitosan scaffold were analyzed using the Kolmogorov-Smirnov test to determine the normality of the data distribution. The results of the Levene statistic test on day 1 on the negative control, positive control, and treatment obtained a value of 0.075 ($p>0.05$); the third day on the negative control, positive control, and treatment obtained a value of 0.002 ($p>0.05$); day 7 on the negative control, positive control, and treatment obtained a value of 0.228 ($p>0.05$); Day 14 on negative control, positive control, and treatment obtained a value of 0.297 ($p>0.05$), indicating that the data is normally distributed as shown in table 7.

Table 5 Kolmogorov-Smirnov Test Results Serum Calcium Levels for Negative Control, Positive Control, and Treatment for various days showed a significance value of p> 0.05.

	N	Sig.
Day 1 negative control	4	0,586
3rd day negative control	4	0,836
7th day negative control	4	0,586
14th day negative control	4	0,816
Day 1 positive control	4	0,431
3rd day positive control	4	0,553
7th day positive control	4	0,436
Positive control day 14	4	0,301
1st day treatment	4	0,345
3rd day treatment	4	0,742
7th day treatment	4	0,548
14th day treatment	4	0,549

Table 6 Results of the Levene Statistic Homogeneity Test obtained p>0.05.

		Sig.
Day-1 Ca	**Negative Control**	**0,075**
	Positive Control	
	Treatment	
Day-3 Ca	**Negative Control**	**0,002**
	Positive Control	
	Treatment	
Day-7 Ca	**Negative Control**	**0,228**
	Positive Control	
	Treatment	
Day-14 Ca	**Negative Control**	**0,297**
	Positive Control	
	Treatment	

The ANOVA test table found a significance value of 0.000 (p<0.05), which means that there is a significant difference in each treatment group (Table 7).

Table 7 ANOVA Test Results for Serum Calcium Levels in Wistar Rats on hADMSCs and Chitosan Scaffold.

		Sig.
Hari ke-1 Ca	**Negative Control**	**0,000**
	Positive Control	
	Treatment	
Hari ke-3 Ca	**Negative Control**	**0,000**
	Positive Control	
	Treatment	
Hari ke-7 Ca	**Negative Control**	**0,001**
	Positive Control	
	Treatment	
Hari ke-14 Ca	**Negative Control**	**0,000**
	Positive Control	
	Treatment	

In the Post Hoc test using Tukey HSD, it was found that there were significant differences between the negative control group, the positive control group, and the treatment group on days 1, 3, 7 and 14. There were significant differences between the treatment group on day 1 and all groups. control also with the treatment group on day- ($p<0.05$).

DISCUSSION

The use of Human Adipose-derived Mesenchymal Stem Cells (hADMSCs) in this study is based on the successful use of hADMSCs in clinical trials conducted from 2004 to 2014. The application of hADMSCs to bone tissue requires a scaffold so that the hADMSC tissue does not denature. Chitosan scaffold was chosen because chitosan scaffold has a similar structure to glycosaminoglycans (Thein-Han et al., 2008).

Based on the results of the study, a one-way ANOVA test was carried out to determine there was a significant difference between each group ($p<0.05$). The results of the one-way ANOVA test in this study found a significance value of 0.000 ($p<0.05$), which means that the comparison of serum calcium in each group in the negative control group, the positive control group and the treatment group on days 1, 3, 7 and 14 there is a difference. significant.

On the first day of negative control, serum calcium levels increased due to the inflammatory process. Bone fractures are recognized by the body as a stressor. In the brain, stressors are interpreted as a host defense response which will then stimulate the hypothalamus and activate the hormonal system of the HPA (Hypothalamic-pituitary-adrenal) axis. Activation of the HPA

axis pathway will stimulate the hypothalamus to secrete corticotropic releasing hormone (CRH). CRH stimulates the anterior pituitary to secrete ACTH (adrenocorticotropic hormone) then ACTH will trigger the adrenal cortex to secrete glucocorticoid hormones. One of the glucocorticoid hormones that are secreted when there is stress is corticosterone. Increased levels of corticosterone will increase the activity of inflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (Ghiasi et al., 2017; Oryan et al., 2012) which activates osteoclasts. Then the osteoclasts will resorption bone resulting in the release of extracellular calcium. The release of calcium minerals into the extracellular will have an impact on increasing serum calcium levels (Allori et al., 2008).

Immediately after bone fracture, hematoma formation occurs due to rupture of blood vessels. The tissue in the fracture area will experience hypoxia due to reduced blood supply due to damaged blood vessels (Oryan et al., 2012) which then encourages bone resorption due to decreased secretion of PTH (Parathyroid hormone). The decrease in PTH on the first day may occur due to bone immobilization or hemiplegic stroke (Sato et al., 2010) which can lead to increased bone resorption, decreased bone formation and increased serum calcium levels (Papavasiliou et al., 2012).

The mean serum calcium level in the control group on the first day of treatment had the lowest value compared to the negative control group and the positive control group because mesenchymal stem cells can be induced to become osteoblasts if there are inflammatory cytokines such as IL-1, IL-6 and TNF- (Sonomoto et al., 2012; Tanaka, 2015). Thus, on the first day of acute inflammation where the activity of IL-1, IL-6 and TNF-α is high, the treatment group given hADMSCs and chitosan scaffold can induce mesenchymal stem cells to become osteoblasts more quickly.

Then on the third day, proliferation and differentiation from pre-osteoblasts to osteoblasts began. The activity of IL-1, IL-6 and TNF-α is also suppressed so that osteoclast production decreases and bone resorption also decreases but the inflammatory process still occurs (Dimitriou, 2005).

On the seventh day the mean serum calcium level increased compared to the third day, which means the extracellular Ca2+ increased. This is because on the seventh day is the peak of TGF-β (Dimitriou, 2005) which functions to increase proliferation, angiogenesis and formation of connective tissue and soft callus via intramembranous and endochondral ossification by inhibiting osteoclast activity (Ghiasi et al., 2017; Goldhahn et al., 2012; Marsell & Einborn, 2011).

On the seventh day of the treatment group where hADMSCs and chitosan scaffold were added, serum calcium levels experienced the highest increase compared to the negative control group in the positive control group because hADMSCs were able to increase TGF-β levels so

that the proliferation process from undifferentiated mesenchymal stem cells to osteoblasts and chondroblasts increased (Chen et al., 2012).

On the fourteenth day the mean serum calcium level increased both in the negative control group, the positive control group, and the treatment group compared to the seventh day. This is because the fourteenth day is the most active phase in osteogenesis which will then continue until day 21 (Dimitriou, 2005). In animals, the fourteenth day is the peak of hard callus formation (Marsell & Einborn, 2011). On the fourteenth day there was resorption of hard callus by osteoclasts and formation of woven bone by osteoblasts through bone remodeling mechanism (Marsell & Einborn, 2011). Research using in vivo animal study models is important to find out more about the mechanisms that occur after the in vitro study model, but pain, stress and death experienced by animals are important issues that are considered by many researchers (Dhoke and Dhawale, 2015) .

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