Levels of Wistar Calcium Serum (*Rattus norvegicus*) in Human Adipose-derived Mesenchymal Stem Cells (hADMSCs) and Chitosan Scaffold by Osteoinduction Examination

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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, stem cells that have self-renewal ability and multilineage differentiation, bioreactor / growth factor, and scaffold. The combination of hADMSCs and chitosan scaffold, is expected to trigger osteoinduction shown by osteogenic markers such as calcium levels.

Objectives to prove osteoinduction activity in a combination of Human Adipose-derived Mesenchymal Stem Cell (hADMSCs) and chitosan scaffold using serum calcium levels.

There were 12 treatment groups with each group having 4 samples. Groups 1 to 4 were the negative control group at day one, three, seven, and fourteen which maxillary bone drilling only. While groups 5 to 8 were the positive control group at day one, three, seven, and fourteen which were given chitosan scaffold. Groups 9 to 12 were treatment group at day one, three, seven, and fourteen which were given hADMSCs and chitosan scaffold. Blood is collected in each group to check serum calcium levels. There were differences in calcium levels in blood serum in each group.

The application of hADMSCs and chitosan scaffold caused a significant change in serum calcium levels on the day one, three, seven, and fourteen which meant that the combination of hADMSC and chitosan scaffold could trigger osteoinduction.

Experimental article (J Int Dent Med Res 2023; 16(1): 20-23)

Keywords: human adipose-derived mesenchymal stem cells, chitosan scaffold, tissue engineering, calcium, patient satisfaction.

Received date: 21 August 2022 **Accept date:** 10 September 2022

Introduction

Extensive reconstruction of bone tissue requires a bone graft. Autografts are "Gold standards" that are used for bone tissue reconstruction to date. However, autografts have only produce limited number of cell.[1] So, new method is needed to augment extensive bone defects.

Tissue engineering is the latest method of regenerating tissue without leaving scar.[2] Tissue Engineering has three main components, cells, scaffold, growth factor or bioreactor.[3,4,5] Stem cells have the ability to self renewal and multilineage differentiation.[6] Bone marrow mesenchymal stem cells (BMSCs) is a source of stem cells commonly used in tissue engineering.[7] BMSCs are able to differentiate into chondrocytes, osteoblasts, adipocytes, fibroblasts and other mesenchymal tissues.[8] However, BMSCs produce a low number of stem cells, require more in vitro time, painful aspiration procedures, poor proliferation and cell osteogenic abilities are influenced by the age of the patient. The source of new stem cells is found from fat tissue, so-called adipose-derived stem cells (ASCs) and when obtained from humans it is called human adipose-derived mesenchymal stem cells (hADMSCs).[9] hADMSCs are considered more suitable in clinical applications because the number of stem cells produced through lipo-aspiration is numerous, cell

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proliferation is fast, aspiration procedures are more comfortable and death during lipoaspiration is lower.[10,11] However, the osteogenic capacity of hADMSCs is not greater than that of BMSCs.[12] Therefore, a material is needed to increase the osteogenic capacity of hADMSCs.

Bone regeneration requires osteoconductive, osteoinductive scaffold while providing a suitable microenvironment for proliferation, differentiation and tissue formation.[13] Some of the scaffolds used today are collagen, chitosan, gelatin, alginate, fibrinogen, and hyaluronic acid.[14] Chitosan is chosen as scaffold because chitosan has biocompatible properties, can mimic the original network microenvironment and chitosan can increase cell growth and mineral deposition of minerals by osteoblast cells.[15] In addition, chitosan scaffold also has a structure similar to glycosaminoglycan. Glycosaminoglycan is one of the main components connected with collagen fibers in the extracellular matrix (ECM).[16]

Objectives to explain bone osteoinduction activity that can be seen by osterix (OSX) expression and serum calcium level as evidenced by the active mechanism of sending induction stem cell signals to differentiate into pre-osteoblasts.[17]

Materials and methods

Research Samples

This study used a sample of chitosan scaffold in the form of membrane with porosity 25 taken from stock at BATAN and hADMSCs taken from human adipose tissue at the Airlangga University Stem Cell Research and Development Center. Male wistar (Rattus Norvegicus) rats aged 3 months with a body weight of 300-330 grams as many as 48 birds with healthy conditions were obtained from UD. Tiput Abadi Jaya. The sample size in each group was calculated using the Federer formula and obtained at least 3 rat samples each group.

Research Methods

Anesthetic injection using ketamine HCl 50 mg / Kg BB and xylazine HCl 10 mg / Kg BB intramuscularly in the femoral caudal extremity. Then maxillary bone drilling was done, 1 cm below the eye using a low-speed hand piece operated 1500 rpm lateral to 3 mm long and profundus as long as 2 mm. During drilling sterile saline solution was given.

In the first group, the negative control group only performed maxillary bone drilling and were not given hADMSCs or chitosan scaffold but saline was added during drilling. The second group was the positive control group performed maxillary bone drilling and was given chitosan scaffold after bone drilling and addition of saline during drilling. The third group, the treatment group, carried out maxillary bone drilling by adding hADMSCs on chitosan scaffold after bone blast and adding saline during drilling.

On the day one, three, seven, and fourteen 2 cc of blood was collected in the aorta. Next, blood was put into a centrifugation tube and centrifuged 1500 rpm for 20 minutes to obtain serum. The serum is labeled according to group and stored in a deep freezer at -70^{o}C until all serum samples are collected. Measurement of calcium levels is carried out by the principle of calorimetry method O-cresolphthalein complexone. A serum sample of 1 ml piped into the test tube was then added with 4 ml of 5% TCA. The solution is cortexed (homogenized), then centrifuged at 3000 rpm for 30 minutes. The resulting supernatant was pipetted 1 ml each into the test tube, then added a solution of strontium (Sr) 5% as much as 1 ml and added as much as 8 ml of distilled water. After that, it was analyzed by a spectrophotometer at a wave of 422.4 nm. The standard calcium solution used is calcium carbonate ($CaCO_3$). The reading results are then compared to the standard curve, so that calcium levels are obtained in units of mg / dl or ppm 28.

Ethical clearance had been obtained from the Ethics Commission of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya (No. 255 / HRECC.FODM / IX / 2018) on 18 September, 2018.

Statistical methods

The results of the next study were analyzed statistically by one way ANOVA and continued with the Tukey HSD test with a significance value of $p < 0.05$.

Results

The results showed that calcium levels changed in the treatment group when compared to the negative control group and the positive control group both on day one, three, seven, and fourteen.

On the day one serum calcium levels in the treatment group had the lowest value compared

to the negative control group and the positive control group (Table 1).

	Group	Mean ± SD	F	P value
Day one	Negative Group[a]	10.2075 ± 0.11587	59.548	0.000
	Positive Group[b]	12.05 ± 0.58023		
	Treatment Group[c]	9.4775 ± 0.963		
Day three	Negative Group[a]	9.99 ± 0.207	45.438	0.000
	Positive Group[b]	11.85 ± 0.591		
	Treatment Group[c]	9.4375 ± 0.170		
Day seven	Negative Group[a]	9.59 ± 0.146	18.868	0.001
	Positive Group[b]	12.05 ± 0.619		
	Treatment Group[c]	11.65 ± 0.838		
Day fourteen	Negative Group[a]	9.92 ± 0.294	113.519	0.000
	Positive Group[b]	12.75 ± 0.12910		
	Treatment Group[c]	12.575 ± 0.40311		

Table 1. Mean, standard deviation (SD), and p value of serum calcium of the negative, positive, and treatment group. The different superscript letters are statistically different (ANOVA, $P < 0.05$).

Whereas on the day three, the serum calcium level in the negative group and the treatment group had an average value that was not far adrift. The mean serum calcium levels in both the negative, positive and treatment groups on the third day decreased slightly compared to the day one (Table 1).

On the day seven the mean of negative, positive and treatment groups are increased compared to the day three. The serum calcium level of the treatment group has the highest increase compared to the negative control group and the positive control group (Table 1).

On the day fourteen, the mean serum calcium levels in the negative, positive, and treatment groups increased compared to the day seven (Table 1).

The results of one way ANOVA test in this study found a significance value of $p = 0.000$ ($p < 0.05$) which means the comparison of serum calcium levels in each group in the negative control group, the positive control group and the treatment groups day one, three, seven, and fourteen were the difference with the value of $p = 0.000$ ($p < 0.05$). Furthermore, to find out further differences between groups, the Tukey HSD test was conducted. Tukey HSD results showed that there were significant differences between groups in both the negative control group, the positive control group and the treatment group (Table 1).

Discussion

Tissue engineering is a cutting-edge strategy that aims to restore the function of the tissue that has a defect. Tissue engineering has three components, namely growth factor or bioreactor, biomaterial scaffold, and cell. Absolute bone regeneration process requires osteoconductive, osteoinductive scaffold while providing a suitable microenvironment for proliferation, differentiation and tissue formation.[18]

On the day one of negative control, serum calcium levels increased due to the inflammatory process. Bone fractures are known by the body as stressors. In the brain, stressor is translated as a host defense response which then stimulates the hypothalamus and activates 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 secreted when stress occurs is corticosterone. Increased corticosterone levels will increase the activity of inflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α)[19,20] which activate osteoclasts. Then osteoclasts will resorb bone resulting in extracellular calcium release. The release of calcium to extracellular minerals will have an impact on increasing serum calcium levels1.[21]

Immediately after a bone fracture, hematoma formation results from rupture of a blood vessel. Tissue in the fracture area will experience hypoxia due to reduced blood supply due to damage to blood vessels[22] which then encourages bone resorption due to a decrease in PTH secretion (Parathyroid hormone). PTH is an important hormone secreted by the parathyroid gland which functions to regulate blood calcium levels.[23]

The decrease in PTH on the day one is likely due to bone immobilization or hemiplegic

stroke[24] which can cause increased bone resorption, decreased bone formation and increased serum calcium levels.[23,25]

The mean serum control serum calcium level had the lowest value compared to the negative control group and the positive control group because mesenchymal stem cells could be induced to become osteoblasts if there were inflammatory cytokines such as IL-1, IL-6 and TNF-α.[26,27] Thus, on the first day of acute inflammation where IL-1 IL-6 and TNF-α activity was high, the treatment group given hADMSCs and chitosan scaffold could more quickly induce mesenchymal stem cells to become osteoblasts. Besides mesenchymal stem cells can produce osteoprotegerin which serves to suppress RANKL / RANK pathways so that the osteoclastogenesis process is inhibited[28] and extracellular Ca^{2+} decreases. The decrease in extracellular Ca^{2+} results in a decrease in serum calcium levels.

Then on the day three proliferation and differentiation of pre-osteoblasts becomes osteoblasts. The activity of IL-1, IL-6 and TNF-α was also suppressed so that the production of osteoclasts decreased and bone resorption also decreased but the inflammatory process still occurred.[6] This is what causes the serum calcium level on the day three to decrease compared to the day one. In the treatment control, serum calcium levels decreased slightly compared to the day one because of the inflammatory process that still occurs so that the presence of IL-1, IL-6 and TNF-α in bone healing process causes mesenchymal stem cells hADMSCs to bind IL-1, IL-6 and TNF -α through the WnT pathway signaling and osteoclast production decreases.[29,30]

On the day seven the mean serum calcium level increases compared to the third day which means extracellular Ca^{2+} rises. This is because on the seventh day is the peak of TGF-β 6 which functions to increase proliferation, angiogenesis and formation of connective tissue and soft callus via intramembran and endochondral ossification by inhibiting the activity of osteoclasts.[15,31,32] Meller et al., Also explained that increased PTH levels were seen during the formation of soft callus as compensation for the body to mobilize calcium from bone as a callus precursor mineralization.[33,34] So that when PTH levels increase, calcium in plasma levels also increase because PTH decreases osteoclast activity, increases osteoblast maturation and decreases collagen osteoblasts in the bone matrix.

Whereas in the day seven treatment group where hADMSCs and chitosan scaffold were added the serum calcium level had the highest increase compared to the negative control group, the control group was positive because hADMSCs were able to increase TGF-β levels so that the proliferation of undifferentiated mesenchymal stem cells increased osteoblast and chondroblast cells. In addition, hADMSCs also increase the production of TGF-β1 which increases osteoblast differentiation and reduces the ability of osteoblasts to osteoclast secretion by releasing OPG to inhibit osteoclast function for bone resorption so that bone mineralization processes can increase.[5] Thus the serum calcium level on the seventh day of the treatment group increased.[30,31]

The day fourteen of the serum calcium level increased in both the negative control group, the positive control group, and the treatment group compared to the day seven. This is because the fourteenth day is the most active phase of osteogenesis which will then continue until the day twenty one. In animals, the fourteenth day is the peak of hard callus formation.[18] Hard callus is a rigid and stable structure but does not yet have perfect biomechanical abilities like normal bones. Therefore on the day fourteen there is hard callus resorption by osteoclasts and the formation of woven bone by osteoblasts through a bone remodeling mechanism.[18] In this phase there is an increase in TNF-α, TNF-α indirectly inactivated osteoclasts through the production of IL-1 and IL-6 which will then encourage the occurrence of osteoclastogenesis.[1] The activity of osteoclastogenesis will result in increased extracellular calcium, therefore the serum calcium level in the blood is higher.[34]

Through this study, it can be proven that the application of Human Adiposed Derived Mesenchymal Stem Cells (hADMSCs) and chitosan scaffold can cause significant changes in calcium levels in blood serum both on day one, three, seven, and fourteen. This indicates that this vivo application of Human Adiposed Derived Mesenchymal Stem Cell (hADMSC) and chitosan scaffold can lead to an osteoinduction process.

Conclusions

The application of hADMSCs and chitosan scaffold caused a significant change in serum calcium levels on the day one, three, seven, and fourteen which mean that the combination of hADMSC and chitosan scaffold could trigger osteoinduction.

Acknowledgements

The authors are grateful to the Indonesian Ministry of Research and Technology and the Faculty of Dental Medicine, Universitas Airlangga, Surabaya for the funding support so this research could be conducted.

Declaration of Interest

The authors report no conflict of interest.

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