Osteoinduction Ability of Human Adipose-Derived Mesenchymal Stem Cell with Chitosan Scaffold Combination Towards Blood Serum Phosphorus Levels

Dian Agustin Wahjuningrum[1], Nindya R A Marhendra[2], M. Roelianto[1], Ari Subiyanto[1], Irmaleny[3], Fery Setiawan[4], Syania E Febriyanti[2], Setyabudi[1]*, Tamara Yuanita[1], Swadheena Patro[5], Anuj Bhardwaj[6,7]

1. Department of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia
2. Resident of Department Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia
3. Department of Conservative Dentistry, Faculty of Dentistry, Universitas Padjajaran, Bandung, Indonesia
4. Doctoral Student, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
5. Department of Conservative and Endodontics. Faculty of Dentistry. Kiit University. India
6. Adjunct Professor at Department of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia
7. Department of Conservative Dentistry and Endodontics, College of Dental Science and Hospital, Rau, Indore.

Abstract

Reconstruction of extensive bone tissue damage is a treatment with complication. Moving the autologous tissue can cause problems in the repair of extensive tissue damage, so the principle of tissue engineering is used as an alternative to reconstruct damage to the tissue because it has many advantages. The combination of Human Adipose-Derived Mesenchymal Stem Cell (hADMSC) and chitosan scaffold is expected to trigger osteoinduction that can be expressed by osteogenic markers such as phosphorus levels in blood serum.

Objectives to prove osteoinduction in a combination of hADMSC and chitosan scaffold using blood serum phosphorus levels. This study used 12 groups with 5 sample each. Groups 1 to 4 were the negative control group at day 1,3,7, and 14. While groups 5 to 8 were the positive control group at day 1,3,7, and 14. Groups 9 to 12 were treatment groups at day 1,3,7, and 14. In the negative control group bone was only removed, in positive control group, bone was removed and chitosan scaffold was added, and in treatment group, bone was removed then, hADMSC and chitosan scaffold combination was added.

Blood collection will be carried out in each group for examination of phosphorus levels in the blood serum. There were differences in phosphorus levels in blood serum in each group even though statistically there were only significant differences on day 14. The combination of hADMSC and chitosan scaffold caused a significant change in blood serum phosphorus levels on day 14 which means it triggers osteoinduction.

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Introduction

Background

Tissue engineering is the application of the principles and methods of a homeostasis technique and science to a basic understanding of structural and functional relations in normal and pathological mammalian networks as well as the development of biological replacement to restore, maintain, or improve tissue function[1]. Tissue engineering is used in regenerative medicine which aims to repair and replace damaged or lost tissue by initiating natural regeneration processes such as osteoinduction which plays an important role in bone tissue regeneration[2,3].

Reconstruction of extensive bone tissue damage is one of the most difficult treatments for operators today[4]. The gold standard for improving bone repair is transplantation of fresh autologous bone[5]. However, when transferring autologous tissue such as bone graft it can cause complications because of the limited supply of

Corresponding author:
Setyabudi.
Faculty of Dental Medicine, Universitas Airlangga
St. Mayjen Prof. Dr. Moestopo No. 47, Surabaya, Indonesia
E-mail: setyabudi@fkg.unair.ac.id

tissue that causes problems with extensive tissue damage repair[4,5]. The principle of tissue engineering is used as an alternative to reconstruct damage to tissue because it has many advantages, namely, decreased donor morbidity, decreased procedural sensitivity in repair, and the ability to resemble in vivo tissue environments[4].

The principle of tissue engineering consists of three key elements namely stem cell, bioreactor/growth factor, and scaffold[1]. Stem cells are non-specialized cells that can regenerate themselves over long periods of time with the potential to be able to change into various types of tissues with certain functions[4]. Today it has been investigated that fat is not only an energy reserve but also a source of rich stem cells[6,7]. The benefits of hADMSC are readily available and easily accessed in the body with minimally invasive procedures which are also cheaper and adipose tissue is an operating waste[8,6]. In addition, hADMSC shows stable growth, extensive self-regenerative capacity and the ability to differentiate into various cell lineages such as osteogenic, adipogenic, chondrogenic, hepatogenic, and neurogenic cells, similar to human bone-marrow derived MSCs (BMSCs)[9,7]. In general, hADMSC appears to be a better choice for clinical applications than hBMSCs[10].

Furthermore, scaffold is used to become a template or framework of tissue formation and seeded with stem cells[1]. The selection of chitosan as a scaffold because chitosan has been widely used in bone tissue engineering, biocompatible, biomimetics and shows that chitosan increases cell growth and matrix deposition that rich in minerals by osteoblast cells[11]. In addition, the main source of chitosan is crustaceans or crab and shrimp shell waste that are widely found in Indonesia[12].

In this study, by combining hADMSC and chitosan scaffold, it can be used as an alternative to reconstruct damage to bone tissue by regenerating its original tissue. The presence of osteoinduction in combining the two ingredients, it is shown by several osteogenic markers such as, RUNX2 (run-related transcription factor 2) and phosphorus[13,14]. RUNX2 is osteoblast-related genes important in differentiating osteoblasts as well as chondrocyte maturation. RUNX2 induces chondrocyte maturation and increases chondrocyte proliferation through direct induction by expression of IHH (Indian Hedge Hog) which will induce PTHrP (Parathyroid-related Hormones Pathway)[13]. IHH and PTHrP will also express RUNX2 in the perichondrium and induce osteoblast differentiation[13]. Phosphorus or what can be called phosphate is one of the most abundant minerals in the human body and plays an important role in bone mineralization processes[14,15]. The serum phosphorus concentration is regulated by PTH (Parathyroid Hormones) which will increase phosphorus levels in blood serum during bone resorption[16]. Therefore, the osteoinduction of hADMSC with chitosan scaffold should be examined using RUNX2 markers as well as phosphorus levels in blood serum. This study was carried out in-vivo with a combination of adipose derived mesenchymal stem cells taken from human adipose tissue seeded in chitosan scaffold in the maxillary bone of wistar rats and examined using RUNX2 and phosphorus markers in blood serum as osteoinduction markers.

Materials and methods

Research Samples
The experimental animals used in this study were Wistar (Rattus norvegicus) rats, aged 3-month-old with an initial body weight of 300-330 gr, obtained from Gajah Mada University, Yogyakarta, Indonesia. The Wistar rats (Rattus norvegicus) were housed in a cage made of 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 with free access to food and water.
Research Methods
1. Sample preparation stage
Experimental animals are randomly selected and divided into 12 groups: The K (-) 1 group is the negative control group as normal bone which is drilled. Phosphorus levels in blood serum were checked on day one, The K (-) 3 group is the negative control group as normal bone which is drilled. Phosphorus levels in blood serum were checked on the day three, The K (-) 7 group was the negative control group as a normal bone which is drilled. Phosphorus levels in the blood serum were checked on the day seven, The K (-) 14 group was the negative

control group as a normal bone which is drilled. Phosphorus levels in blood serum were checked on day fourteen. The K (+) 1 group is the positive control group with the administration of chitosan scaffold after bone drilling. Phosphorus levels in blood serum were checked on day 1. The K (+) 3 group was the positive control group with the administration of chitosan scaffold after bone drilling. Phosphorus levels in blood serum were checked on the day three. The K (+) 7 group was the positive control group with the administration of chitosan scaffold after bone drilling. Phosphorus levels in the blood serum were checked on the day seven. The K (+) 14 group was the positive control group with the administration of chitosan scaffold after bone drilling. Phosphorus levels in blood serum were checked on day fourteen. The treatment group P1 is the group with the administration of hADMSC on chitosan scaffold after bone drilling. Phosphorus levels in blood serum were checked on day one. The treatment group P3 is the group with the administration of hADMSC on chitosan scaffold after bone drilling. Phosphorus levels in blood serum were checked on the day three. The treatment group P7 is the group with the administration of hADMSC on chitosan scaffold after bone drilling. Phosphorus levels in blood serum were checked on day seven. The treatment group P14 is the group with the administration of hADMSC on chitosan scaffold after bone drilling. Phosphorus levels in blood serum were checked on day fourteen.

2. Human Adipose-derived Mesenchymal Stem Cell isolation and culture

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). Infranatant 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)[22]. 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[23].

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 Flow cytometer (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 DMSO 200 microliter/well. Each the well read by Elisa Reader at a wavelength of 595 nm.

4.   hADMSC seeding in chitosan scaffold

Cell suspension containing 5×10^5 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 body weight 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 kalium 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.   Phosphorus serum study

All groups of experimental animals were given uniform standard feed during adaptation until the end of the treatment. On day one, three, seven and fourteen, 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 phosphorus level were examined using UV-VIS method.

Statistical methods

Data of all phosphorus levels were firstly analyzed using the One-Sample Kolmogorov-Smirnov test to find out the normal distribution using p value = 0.05. After checking the normality test all the groups had P<0.05 so that oneway Anova statistical test can be done to find out the significant difference using p value = 0.05. The phosphate on the day one, three, and seven were insignificant with P>0.05, but the phosphate on the day fourteen was significant with P<0.05. Thus, it can be stated that there was a significant difference data in the phosphate level on the day fourteen among the day one, three, and seven.

Results

This study was conducted *in-vivo* with the combination of human adipose derived mesenchymal stem cells taken from human adipose tissue seeded in chitosan scaffold in the maxillary bone of wistar rats that have been drilled. The chitosan scaffold toxicity test for human adipose derived mesenchymal stem cells using the MTT assay method was carried out at the Airlangga University Stem Cell Research Center. Observation of the ability of combined osteoinduction of human adipose derived mesenchymal stem cell and chitosan scaffold was carried out in 12 groups by measuring the phosphorus levels in rat blood serum from each sample and expressed in units (mg/dl) in tables 1, 2, 3, and 4.

Sample	Day One		
	Negative Control (K (-) 1) (mg/dl)	Positive Control (K (+) 1) (mg/dl)	Treatment (P1) (mg/dl)
1	7	9	9.1
2	7.5	9.1	10.9
3	7.9	7.2	6.7
4	8	5.3	8.7
Mean	7.6	7.65	8.85

Table 1. Phosphorus levels in blood serum in day one.

On the first day (table 1), the average number of phosphorus levels with the difference in each group was 7.6 mg/dl in the negative control, 7.65 mg/dl in the positive control and 8.85 mg/dl in the treatment. On the third day (table 2), the number of phosphorus levels are higher in the positive control group was 8.6 mg / dl, whereas in the negative control the phosphorus level was 6.725 mg/dl and the treatment showed phosphorus levels of 6.6 mg/dl.

Sample	Day Three		
	Negative Control (K (-) 3) (mg/dl)	Positive Control (K (+) 3) (mg/dl)	Treatment (P3) (mg/dl)
1	6	8.3	4.8
2	6.8	8.6	7
3	6.2	6.2	6.7
4	7.9	9.2	7.9
Mean	6.725	8.6	6.6

Table 2. Phosphorus levels in blood serum in day three.

Sample	Day Seven		
	Negative Control (K (-) 7) (mg/dl)	Positive Control (K (+) 7) (mg/dl)	Treatment (P7) (mg/dl)
1	9.7	9	8.2
2	8.8	8.8	8.8
3	9.7	6.6	8.4
4	9.4	7	6.6
Mean	9.4	7.85	8

Table 3. Phosphorus levels in blood serum in day seven.

Sample	Day Fourteen		
	Negative Control (K (-) 14) (mg/dl)	Positive Control (K (+) 14) (mg/dl)	Treatment (P14) (mg/dl)
1	8	8.4	6.4
2	8.6	6.8	9
3	9.8	5.6	7
4	9.6	6.4	6
Mean	9	6.8	7.1

Table 4. Phosphorus levels in blood serum in day fourteen.

On the day seven (table 3) there was a higher number of phosphorus levels in the negative control ie 9.4 mg/dl. In the positive control, phosphorus levels were 7.85 mg/dl and phosphorus levels were 8 mg/dl. Then, on the day fourteen (table 4) found that the phosphorus level in the negative control was 9 mg/dl, in the positive control there was a 6.8 mg/dl phosphorus level, and the treatment was 7.1 mg/dl phosphorus.

	Group	Mean ± SD	P value
Phosphate day one	Negative Control (K (-) 1)	7.6 ± 0.45461	0.426
	Positive Control (K (+) 1)	7.65 ± 1.79351	
	Treatment (P1)	8.85 ± 1.72337	
Phosphate day three	Negative Control (K (-) 3)	6.725 ± 0.85391	0.199
	Positive Control (K (+) 3)	8.0750 ± 1.30480	
	Treatment (P3)	6.6 ± 1.30384	
Phosphate day seven	Negative Control (K (-) 7)	9.4 ± 0.42426	0.082
	Positive Control (K (+) 7)	7.85 ± 1.22610	
	Treatment (P7)	8 ± 0.96609	
Phosphate day fourteen	Negative Control (K (-) 14)	9 ± 0.84853[a]	0.046
	Positive Control (K (+) 14)	6.8 ± 1.17757[b]	
	Treatment (P14)	7.1 ± 1.33167[c]	

Table 5. Mean and standard deviation of each group based on phosphate day one, three, seven, and fourteen. The different superscript letters are statistically significant (Tukey HSD, P < 0.05).

Table 3 shows that the negative control group on the day seven showed the highest serum phosphorus level compared to the other groups, while the lowest serum phosphorus level was the treatment group the day three in table 2.

Data from this study in the form of phosphorus levels need to be analyzed first using the One-Sample Kolmogorov-Smirnov test to find out the normal distribution of the results of the study and the test of homogenity of variances to determine the homogenity of the results of the study. After doing the two statistical tests, oneway Anova statistical test can be done to find out the differences between sample groups. Based on the results of the normality of serum phosphate levels using the one sample Kolmogorov-Smirnov Test, it is known that the data is normally distributed because it has significance value > 0.05. Based on the test results of homogenity of serum phosphate levels,

it is known that the overall data is homogeneous because it has significance value > 0.05.

Oneway Anova test was used to determine the significant differences in all groups. From this test, the value of P or significance will be obtained between groups and all groups in table 5. On the day one, day three, day seven showed a p value greater than the significance value (p> 0.05) so that the phosphate level can be concluded on the day one, three and seven were not significant. However, on day fourteen, the test results showed a p value smaller than (p <0.05) which could be concluded that the phosphate level on day fourteen had a significant value between groups.

Discussion

This study was conducted *in vivo* to prove the presence of changes in phosphorus levels in blood serum as an indication of osteoinduction after transplantation of Human Adiposed Derived Mesenchymal Stem Cell (hADMSC) with chitosan scaffold on wistar rat maxillary bone defects on day 1,3,7, and 14. Use of Human Adiposed Derived Mesenchymal Stem Cell in accordance with Aksu (2008) which states that Human Adiposed Derived Mesenchymal Stem Cell can differentiate into osteoblast like cells[17]. The use of Chitosan as scaffold in this study is based on Dash (2011) research which states that chitosan increases cell growth and rich minerals matrix deposition by osteoblast cells[11]. So that the combination of the two ingredients will induce an osteoinduction pathway, specifically PTHrP and Indian Hedgehog that will increase RUNX2 to convert hADMSC into osteo-chondroblast progenitors which will later become pre-Osteoblasts. To begin bone remodeling, pre-osteoblast can go through two paths, namely osteoclast formation influenced by PTHrp or the formation of mature osteoblasts which will increase OPG so that it will affect phosphorus levels in blood serum.

From the results of the study conducted an oneway anova test to see statistically whether there were significant differences in results, from statistical analysis showed that the ratio of phosphorus levels in the blood to negative controls, positive control and treatment group on the first, third and seventh days there were no significant differences, because the p value (Sig) shows a number above 0.05. Significant differences between treatment groups were only seen on day fourteen, because the value of p (Sig) showed a number below 0.05.

On the first day, there was no significant difference in the level of phosphorus in the blood because the bone healing process that occur on the first day was an inflammatory process where there was no significant process of resorption and bone mineralization which could affect blood phosphorus levels according to Mountziaris (2008)[18]. But in the results of the study, there were still differences in phosphorus levels from each group, with an mean level of phosphorus in treatment group higher than positive controls and negative controls. The high level of phosphorus in the treatment group can occur because of several possibilities including, dietary phosphate intake is higher than other groups and there is more increase in PTH compared to other groups because the number of Mesenchymal Stem Cells in the treatment group is higher than other groups so that it will increase absorption of phosphate in the intestine[14]. Increased PTH originates from an increase in Ihh-PTHrP axis activity that induces RUNX2 to include hADMSC into the osteoblastic line and osteogenic differentiation of hADMSC which also occurs on the first day of bone healing[19].

Then, on the third day there was also no significant difference in blood phosphorus levels because the bone healing process that occurred was hADMSC proliferation and differentiation of preosteoblasts into osteoblasts which did not significantly affect serum phosphate in the blood according to Mountziaris (2008) and Dimitriou (2005) [18,20]. However, in the results of the study, the lowest levels of phosphorus were found in the treatment group compared to negative controls which might be due to lower dietary phosphate intake compared to other groups and osteogenic differentiation of hADMSC which requires PTH so that PTH decreases which will reduce absorption calcium and phosphate in the intestine[16]. The highest phosphorus levels were found in positive controls which might also caused by changes in the key phosphate regulation in accordance with Penido and Alon (2012) , which are higher dietary phosphate intake than other groups and changes in hADMSC into osteoblastic lines and possible osteogenic differentiation still occurs on day 3 according to Li's study (2015) so that PTH production is still relatively more than normal and can increase phosphate regulation in serum [16, 21].

On the seventh day, there was no significant difference in the level of phosphorus in the blood serum because the normal bone healing process, which occurred on the seventh day was the continuation of the same process from the third day namely the peak of MSCs cell proliferation in intramembranous ossification. So that at this stage there is little effect on phosphate levels in blood serum. From the results of the seventh day study, phosphorus levels in the positive control and treatment were lower than the phosphorus levels in the negative control, which can occur due to lower phosphate dietary intake and the process of changing osteogenic differentiation of hADMSC that requires PTH to be completed.

On the fourteenth day, there is a significant or significant difference in the level of phosphorus in the blood serum. This is because the fourteenth day is the most active phase of osteogenesis until the 21st day is characterized by the cessation of cell proliferation in intramembranous ossification, mineralization of soft callus, cartilage resorption, formation of woven bone, and the beginning of the bone remodeling phase that most affects phosphorus levels in the blood because of the mineralization and resorption processes according to Mountziaris (2008) and Dimitriou (2005)[18,20]. Based on the results of the study, the level of phosphorus in the blood serum in the positive control and treatment was significantly lower than the negative control due to the higher mineralization ratio in the positive control and treatment according to Penido and Alon (2012)[16]. which stated that the increased mineralization would decreases serum phosphorus and calcium concentration, and increased bone resorption will increase serum phosphorus and calcium concentrations. In addition, the low level of phosphorus in blood serum is also due to the increasing number of mature osteoblasts which will allow OPG to inhibit osteoclast function for bone resorption according to the statement of Deschaseaux (2009)[19] so that bone mineralization processes can increase. FGF23 or Phosphatonin which is widely expressed by osteoblasts and osteocytes will also reduce PTH so that it will improve bone mineralization processes in accordance with the statements of Penido and Alon (2012)[16,19].

Conclusions

In conclusion, based on the results of the analysis, the combination of hADMSC and chitosan scaffold caused a significant change in blood serum phosphorus levels on day 14 which means it triggers osteoinduction.

Acknowledgements

Declaration of Interest

The authors declare no conflicts of interest.

Ethical policy and institutional review board statement

Ethical clearance had been obtained from the Ethics Commission of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya ((Permit Number: 217/HRECC.FODM/VIII/2018) in 2018.

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