World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

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Secretion of osteocalcin in chitosan–hydroxyapatite scaffold with seeding of cryopreserved human adipose derived mesenchymal stem cells

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World Journal of Advanced Research and Reviews, 2024, 23(02), 297–304

Publication history: Received on 23 June 2024; revised on 02 August 2024; accepted on 04 August 2024

Article DOI: https://doi.org/10.30574/wjarr.2024.23.2.2314

Abstract

Background: Tissue engineering has developed in recent years, including the utilization of bone graft to regenerate bone. The incorporation of chitosan and hydroxyapatite as scaffolds improves osteoconductive ability. Human adipose derived mesenchymal stem cells (hADMSCs) potentially differentiate into osteoblasts and can accelerate the osteogenesis process.

Purpose: To determine osteocalcin secretion on chitosan-hydroxyapatite (CS-HA) scaffold with seeding of cryopreserved hADMSCs in vitro

Method: Thawing was conducted on frozen (hADMSCs) and characterized using immunocytochemistry. HADMSCs were seeded on CS-HA scaffolds. Examination of osteocalcin secretion levels was carried out by collecting cell culture supernatants of hADMSCs on 7th, 14th and 21st day of the observation group by using osteocalcin ELISA kit.

Result: Scanning observation (SEM) proved that the chitosan-hydroxyapatite scaffold has a suitable pore structure, because hADMSCs can attach and differentiate when attached to the porosity of the scaffold. There were significant differences in osteocalcin secretion on days 7, 14 and 21 according to the ELISA results.

Conclusion: The presence of bone regeneration process in chitosan-hydroxyapatite scaffold. This was characterized by significant osteocalcin secretion on 7th, 14th and 21st day. So it can be concluded that the scaffold is compatible.

Keywords: Bone Tissue Engineering; Chitosan-Hydroxyapatite Scaffold; Osteocalcin; Human Adipose Mesenchymal Derived Stem Cells

1. Introduction

Tissue engineering is a method applied in prosthodontics to restore, recover, and regenerate damaged tissues in prosthodontics. Alveolar fractures are caused by several factors such as post-tooth extraction trauma, periodontal disease, post-enucleation cysts, and post-tumor surgery resulting in decreased bone quality that requires intervention for bone regeneration (1).

Application of tissue-engineered bone graft is a procedure commonly used to restore the function of bone tissue that has been lost or damaged (2). Bone graft improves bone healing response by supporting osteogenic, osteoinductive and osteoconductive properties (3). Scaffolds are one of the solutions for the bone repair process. Scaffold becomes a

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medium for new tissue growth by providing a three-dimensional (3D) structure for cell growth and differentiation, stimulating cell adhesion and cell migration (4).

Osteogenesis is the process of bone remodelling through osteoblast cells. Scaffolds have pores to stimulate cell attachment and differentiation or by combining several biomaterial factors (2). The scaffold acts as a temporary matrix because it has properties similar to the extracellular matrix (ECM) in bone. The scaffold, cell, and signaling components ("Tissue Engineering Triad") are arranged to resemble the natural tissue engineering process

The use of cells in scaffolds induces bone tissue repair and regeneration. The source of cells to develop tissue engineering can be derived from cells of adult tissue origin or stem cells. The hADMSCs are stem cells found in adipose tissue and proven to have multipotency properties to proliferate into adipocytes, osteoblasts, chondrocytes, fibroblasts, and myoblasts. hADMSCs can be collected in high amount through liposuction of subcutaneous adipose tissue fragments and can be easily expanded in vitro and minimally invasive (5).

HADMSCs can be preserved as frozen stock in liquid nitrogen to facilitate storage. Cryopreserved hADMSCs use liquid nitrogen containing cryoprotective agents to delay the freezing process thereby reducing the risk of ice crystals forming that can lead to cell death (6). The cryopreservation method enables the storage or banking procedure of hADMSCs. Cryopreserved hADMSCs produce more abundant hADMSCs, save cost and time, and have the same potential as fresh stock.

The bone remodelling process is observed by examining bone remodelling biomarkers to describe the overall activity of osteoblast and osteoclast cells in bone. Mature osteoblasts will express several chemical compounds that can be used to identify osteoblast activity in serum commonly called biochemical bone markers, including collagen type I, alkaline phosphatase, osteopontin, and osteocalcin.

Increased osteocalcin serum levels associated with increased bone mineral density. Osteocalcin is a γ-carboxyglutamic acid (Gla) bone protein that regulates bone mineralization and biological processes of various organs including bones (7). Osteocalcin is produced during bone formation at the end of the mineralization process. The process of alveolar bone remodelling begins on day 14 with indicators, i.e. the formation of osteoblast cells and bone spicules. On day 21, osteoblast cells and bone spicules are found more. Complete calcification of bone occurs on day 28 (8). The remodelling phase lasts from day 21 to about 1 year. On day 14 osteoblast activity has begun, characterized by the formation of osteoblasts at the periphery of the bone (9).

This study focuses on the bone remodelling process using a CS-HA scaffold seeded by cryopreserved hADMSCs to observe osteocalcin secretion. Osteocalcin levels will increase when osteoblasts have transformed into osteocytes. Therefore, this study will observe osteocalcin secretion.

2. Material and methods

This study is a laboratory experimental study with post-test only control group design. The samples used were CS-HA scaffolds seeded with cryopreserved hADMSCs and then observed for osteocalcin secretion. This study consisted of 3 research groups, including negative control group (hADMSCs + osteogenic medium), positive control (CS-HA scaffold + hADMSCs + alpha mem medium), and experimental group (CS-HA scaffold + hADMSCs + osteogenic medium), each consisting of 3 samples and observed on days 7, 14, 21, and 28.

Cell preparation was begun by thawing the hADMSCs. Cryogenic tubes were removed from liquid nitrogen storage, then immersed in a 37°C water bath. Cells were transferred to a centrifugation tube and then given serum-mixed FBS to protect the outside of the cells during the thawing process so that the DMSO solution did not damage the cells, then centrifuged at 1800 rpm.

Chitosan-hydroxy apatite scaffolds were prepared by dissolving 200mg of medium molecular weight chitosan powder (Sigma-Aldrich, St. Louis, MO, USA) into 5ml of ethanoic acid at room temperature and mixing for 15 minutes. 15 ml of sodium hydroxide solution as a neutralizer to obtain chitosan gel. Next, the chitosan gel sample was mixed homogeneously with 200 mg of HA before centrifuging at 1,500 rpm for ten minutes. After extraction of excess water, the gel solution was placed into a customized mould to produce a scaffold (5 mm diameter and 2 mm height). Before transferring to the drying machine, the gel was frozen for two hours at -80°C.

Next, the seeding process of hADMSCs on the CS-HA scaffold was carried out. The scaffold was inserted into 24 well cell culture plate. Each well requires 3 x 10^4 cells and then added 500µl of α-MEM medium. The process of seeding hADMSCs on the CS-HA scaffold was carried out by immersing hADMSCs in a plate containing α-MEM medium for 1x24 hours until

attached. Osteogenic medium was prepared from conventional culture medium modified by adding 10% FBS, 0.01 μM dexamethasone, 50μg/ml ascorbic acid, 10 mM sodium β-glycerophosphate (Gibco BRL), 10,000U/ml penicillin, and 10,000U/mL streptomycin (Life Technologies).

Osteocalcin secretion was seen through ELISA examination using ELISA Kit (BT LAB) based on Optical Density (OD) readings then collected and processed into mean values and standard deviations. Data processing and data analysis in this study used the Statistical Package for the Social Sciences (SPSS) program consisting of normality test and homogeneity test.

3. Results and discussion

There were three groups in this study, namely positive control, negative control and treatment control groups. The average value and standard deviation will be used to provide descriptive analysis of the data. It will be compared to see if there are differences between groups. Each group on the 7th, 14th and 21st days each had a total of 4 samples for each treatment.

3.1. Scanning Electron Microscope Examination Result

The chitosan-hydroxyapatite scaffold was examined using a Scanning Electron Microscope (SEM). SEM examination was carried out at 500x magnification. SEM examination aims to show that the pore size of the scaffold used in this study is <100μm. SEM examination was also carried out on scaffolds seeded with cryopreserved hADMSCs for 3 days to observe cell attachment to the scaffold.

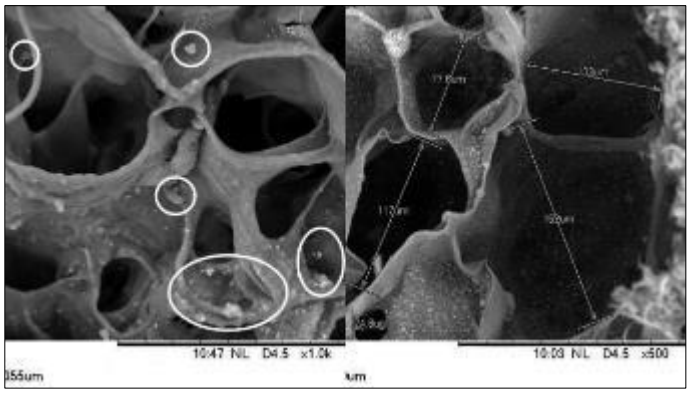

Figure 1 The image shows the attachment of cells to the scaffold, the circled part

3.2. Result of Osteocalcin Secretion on the 17th, 14th, and 21st days

Table 1 Data from the results of the osteocalcin secretion that have not been processed in SPSS.

7th day			14th day			21st day		
PC1	NC1	T1	PC2	NC2	T2	PC3	NC3	T3
47.57	32.57	83.29	53.29	42.57	94.71	61.41	50.67	107.72
54.00	34.71	89.71	40.43	46.14	89.71	59.74	52.61	112.29
61.14	29.57	83.29	52.57	46.14	84.71	52.87	59.01	117.35
46.14	39.71	85.43	58.29	39.00	89.71	53.76	61.32	104.58

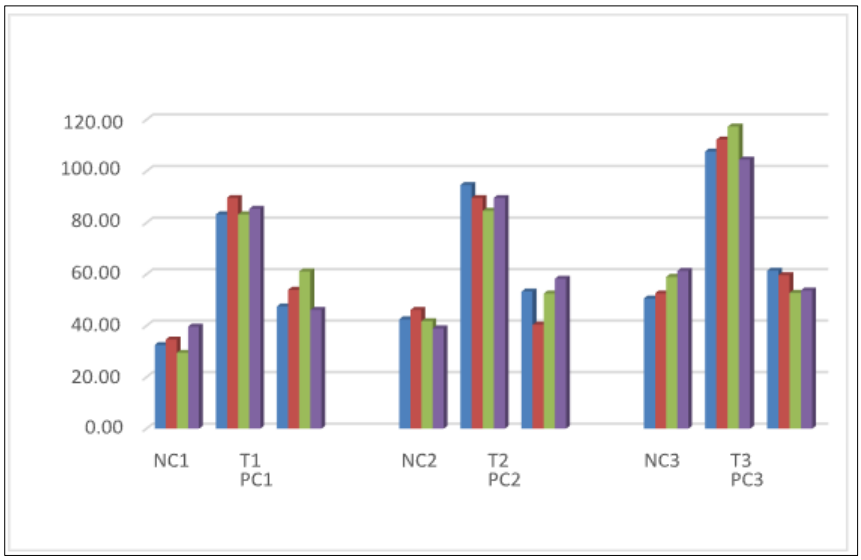

Figure 2 The differences in osteocalcin secretion which have significant differences.

The data will be analyzed descriptively to analyze differences in the amount of osteocalcin secreted by hADMSCs in the osteogenic medium on days 7, 14 and 21 according to the 3 groups. Descriptive analysis uses average and standard deviation. The groups were divided into negative control, positive control and treatment, analyzed descriptively as in table 1. The amount of osteocalcin secretion from differentiated cells was seen through ELISA examination using the ELISA kit (BT LAB). Then the results will be based on optical density (OD) readings with the ELISA tool.

The results of the descriptive analysis showed that in the three groups, bone secretion on day 21 was on average higher than bone secretion on days 7 and 14. Additional statistical analysis was also carried out to determine the extent of the differences between groups. Done using (SPSS).

Table 2 Research data that has been processed with SPSS.

Observation Time (Day)	Groups	Mean±SD
7	PC 1	40.67±3.99
	NC 1	34.14±2.60
	T1	85.43±1.25
14	PC 2	51.14±2.41
	NC 2	43.46±1.35
	T 2	89.71±2.50
21	PC 3	56.25±1.25
	NC 3	55.67±1.41
	T3	110.48±2.81

Based on table 2, it can be seen that the average amount of osteocalcin secretion on day 21 of the three groups has a higher value than the negative control group and the treatment group both on day 7, 14 and day 21. The average value is the highest. than the entire treatment group, namely 110.48 ± 2.81.

3.3. Normality Test for The Amount of Osteocalcin Secretion

The Normality Test was used to determine the differences in osteocalcin secretion in the three groups on day 7, day 14 and day 21. The normality test was analyzed using One-Way ANOVA. The test will be carried out using the Shapiro Wilk normality test.

Table 3 Normality test result

Groups	*Shapiro Wilk*		
	Statistic	df	*p*
PC1	0.827	3	0.515
NC1	0.981	3	0.907
T1	0.929	3	0.589
PC2	0.945	3	0.683
NC2	0.976	3	0.879
T2	0.748	3	0.037
PC3	0.979	3	0.896
NC3	0.910	3	0.483
T3	0.909	3	0.476

Based on table 3, it can be seen that each treatment usually has a p value > 0.05. Thus it can be concluded that H0 is accepted, the data is normally distributed and the normality assumption is met.

3.4. Homogeneity Test for The Amount of Osteocalcin Secretion

Table 4 Homogeneity test result

Groups	*Lavene's Test for Equality of Variances*	
	F	*p*
Osteocalcin secretion	1.069	0.413

The homogeneity test was used to determine that the research data on the amount of osteocalcin secretion on days 7, 14 and 21 had homogeneous data variations. The homogeneity of the data was measured using the Levene Test. From the results of the osteocalcin secretion homogeneity test from table 4, it was found that the three groups had a p of 0.413, this value was greater than the significance level of 0.05 (p > 0.05). It can be concluded that H0 is accepted, the data has homogeneous variations and meets the requirements for using the ANOVA test.

3.5. ANOVA Test for Differences in the Amount of Osteocalcin Secretion

Based on the analysis test results obtained from the previous test, all data met the requirements for homogeneity and normality of the data. Next, it was analyzed using the One-way ANOVA test. This ANOVA test was carried out to determine whether there were significant differences between the six groups. The testing criteria for the statistical test used are the significance value, if the significance value is smaller than 0.05 then H0 is rejected.

Table 5 One Way ANOVA test result

	Sum of Squares	**df**	***Mean Square***	**F**	***p***	
Between Groups	20234.106	8	2529.263	98.049	0.000	Significant differences
Within Group	696.487	27	25.796			
Total	20930.592	35				

The results of the One-way ANOVA test in table 5 show that the difference in the amount of osteocalcin secretion on days 7, 14 and 21 has a p value (0.000) which is smaller than 0.05 (p < 0.05) and H0 is rejected so that It can be concluded that there is a significant difference between the amount of osteocalcin secretion in the six groups. The probability value can also be seen from the F value (98.049) so that H0 is rejected and it can be concluded that there is a significant

difference between the amount of osteocalcin secretion on days 7, 14 and 21. The significant difference can then be analyzed again with a follow-up Post Hoc test Test using Tukey-HSD.

3.6. Post Hoc Test Differences in Osteocalcin Secretion on Days 7, 14, and 21

The results of the One-way ANOVA test showed that there were significant differences between the positive control group, negative control group, and treatment groups on days 7, 14 and 21, so we will then proceed to Post Hoc Test analysis using the Tukey-HSD Test.

Table 3 Tukey-HSD test result

	NC1	**T1**	**PC1**	**NC2**	**T2**	**PC2**	**NC3**	**T3**	**PC3**
NC1		0.000*	0.001*	0.378*	0.000*	0.002*	0.000*	0.000*	0.000*
T1	0.000*		0.000*	0.000*	0.951	0.000*	0.000*	0.000*	0.000*
PC1	0.001*	0.000*		0.183	0.000*	1	0.98	0.000*	0.917
NC2	0.378	0.000*	0.183		0.000*	0.305	0.02*	0.000*	0.01*
T2	0.000*	0.951	0.000*	0.000*		0.000*	0.000*	0.000*	0.000*
PC2	0.002 *	0.000*	1	0.305*	0.000*		0.915	0.000*	0.789
NC3	0.000*	0.000*	0.98	0.02*	0.000	0.915		0.000*	1
P3	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*		0.000*
NC3	0.000*	0.000*	0.917	0.01*	0.000*	0.789	1	0.000*	

Note: (*) Indicates a significant difference ($p<0.05$)

From the results of the Tukey-HSD test in table 6, it is known that the average value of osteocalcin secretion in the positive control group on day 7 has a significant difference from the positive control group on day 21. Meanwhile, the average value of osteocalcin secretion in the treatment group on day 21 had significant differences across groups.

4. Discussion

Extensive developments in the field of tissue engineering have resulted in a range of replacement components and new implementation strategies. Scientific advances in biomaterials, stem cells, growth and differentiation factors, and environmental biomimetics have created unique opportunities to create tissues in the laboratory from combinations of engineered extracellular matrices (scaffolds), cells, and biologically active molecules. One of the biomaterials that has been widely used recently is scaffolds (10).

SEM examination of the chitosan-hydroxyapatite scaffold showed that there was cell attachment to the scaffold wall. This proves the theory that chitosan and hydroxyapatite have high cell attachment, increased pre-osteoblast response, cell proliferation, and good cell distribution in the biomaterial scaffold structure. To achieve the goal of tissue reconstruction, the scaffold must meet several specific requirements. High porosity and adequate pore size are required to facilitate cell seeding and diffusion throughout the cell structure and nutrients. Biodegradability is an important factor because the scaffold should be absorbed by the surrounding tissue without the need for surgical removal. The rate at which degradation occurs must coincide with the rate of tissue formation.

Several important parts of the scaffold, such as pore size, porosity, and pore connectivity, can influence the osteogenic properties of the scaffold. The porosity of the scaffold must be high enough to support cell migration, and nutrient/metabolite exchange (11). Porous scaffolds allow cells to attach and grow into new bone tissue (4).

In this study, frozen (cryopreserved) hADMSCs were used which had gone through a freezing procedure and were stored in liquid nitrogen in DMSO medium containing a cryoprotective agent. This study used cryopreserved hADMSCs because there were no significant differences in the cell population of fresh and frozen hADMSCs. Cryopreserved hADMSCs are easier to use and more efficient to produce high cell viability. Research conducted by Dave *et al* (12) showed that there is little difference between the use of Cryopreserved MSCs and fresh stock, and many researchers prefer Cryopreserved MSCs. Cryopreserved hADMSCs produce more abundant MSCs products, save costs and time, and have the same potential as fresh stock.

Osteocalcin is the most abundant non-collagenous bone matrix protein expressed by osteoblasts. Osteocalcin plays an important role to regulate the mineralization process during osteogenic differentiation of MSCs. Osteocalcin activity is known to increase along with the osteoblast differentiation process. Osteocalcin is produced during bone formation at the end of the mineralization (13). According to research conducted by Ardeshirylajimi *et al.* (14) showed an increase in the expression of osteogenic markers (osteocalcin, RUNX2, ALP, osteonectin) during a 21-day culture period in porous freeze-drying chitosan scaffolds seeded with ADMSCs.

In the research, chitosan-hydroxyapatite scaffold and stem cells in the form of hADMSCs were added to the positive control group and treatment group. From table 5.3.1 the mean amount of osteocalcin secretion in the negative control group tends to be lower than the positive control group and the treatment group. The Tukey Test results table shows that treatment group 3 has a significant value in all groups. The negative control group on day 7 has a significant difference from the treatment group on day 21 (p=0.000).

In the positive control group and treatment group, CS-HA scaffold and stem cells in the form of hADMSCs were added. The mean amount of osteocalcin secretion in the negative control group tended to be lower than the positive control group and the treatment group. In addition, the negative control group on day 14 had significant differences in almost all groups, except for the treatment group on day 7.

Based on the research results, the average amount of osteocalcin secretion in the treatment group on day 21 was the highest and had significant differences in all groups so it can be concluded that the best results were obtained on day 21 with the addition of CS-HA scaffolds and hADMSCs cultured in the medium. osteogenic. According to Grossner et al. (2022) Osteogenic differentiation of MSCs is becoming a standard procedure in modern bone tissue engineering.

5. Conclusion

From this research, the mean amount of osteocalcin secretion produced by the induction of hADMSCs on the chitosan-hydroxyapatite scaffold increased significantly. This showed signs of the osteogenesis process. It is necessary to carry out further research regarding osteocalcin secretion on scaffolds with hADMSCs carried out in experimental animals.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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