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Secretion of osteocalcin in chitosan–hydroxyapatite scaffold with seeding of cryopreserved human umbilical cord mesenchymal stem cells

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Abstract

In recent years, regenerative therapy using tissue engineering has rapidly developed. One of the applications is for alveolar bone tissue engineering in prosthodontics. Currently, the most widely developed biomaterial in bone tissue engineering is a scaffold. The incorporation of chitosan and hydroxyapatite (CS-HA) improves osteoconductive ability. Human Umbilical Cord Mesenchymal Stem Cells (hUCMSCs) are capable of acting as osteoprogenitors and promoting bone formation. Purpose: To determine the influence of osteocalcin secretion in the CS1HA scaffold with seeding of cryopreserved hUCMSCs in vitro. Methods: The thawing process was carried out on frozen hUCMSCs, which were then examined for characterization using immunocytochemistry. The hUCMSCs were seeded on the CS-HA scaffold. Finally, examination of osteocalcin secretion levels was carried out by collecting cell culture supernatant on the 14th and 21st days of observation groups using the human osteocalcin ELISA kit. Results: Immunocytochemistry testing revealed that the hUCMSCs had the characteristics of the MSCs marker, namely positive expression on the CD90 marker and negative expression on the CD45 marker. Scanning electron microscope (SEM) observation revealed that the CS-HA scaffold had a structure with many pores and hUCMSCs could be attached and proliferation among the porosity of the scaffold. Based on the ELISA result, there was a significant difference in the average amount of osteocalcin secretion on days 14 and 21 which indicated osteogenic differentiation. Conclusion: Osteocalcin secretion in the CS-HA scaffold using the seeding of in vitro hUCMSCs demonstrated a bone regeneration process that showed a significant increase on day 21.

Keywords: Bone Tissue Engineering; Scaffold Chitosan-Hydroxyapatite; Osteocalcin; Human Umbilical Cord Mesenchymal Stem Cells

1. Introduction

Alveolar bone is one of the important components in prosthodontic treatment. Having a tooth extracted and not replaced will cause a decrease in alveolar bone mass. In addition, periodontal disease, major trauma after tooth extraction, post-cyst enucleation, and post-surgery can also cause alveolar bone resorption. If not treated properly, within 3 years bone resorption can reach 40-60% of the alveolar ridge volume. One of the efforts to improve this condition is to perform alveolar ridge reconstruction (Sheikh et al., 2015; Rahmitasari, 2016) [1] [2].

Bone graft is one of the most commonly used alternatives to restore the function of bone tissue that has been lost or damaged (Aufan et al., 2012) [3]. According to its origin, a bone graft can be classified into autograft, allograft, xenograft, and alloplastic. A bone graft can improve bone regeneration response by providing osteogenic, osteoinductive, and osteoconductive elements (Amini et al., 2012) [4]. One of the requirements that bone grafts must have in bone tissue engineering applications is the ability to osteogenesis (Mahyudin, 2020) [5]. Osteogenesis or ossification is the process

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of forming bone tissue again through osteoblast cells which includes osteoconduction and osteoinduction (Rather et al., 2019; El Milla et al., 2021) [6] [7]. Currently, biomaterials that are widely developed in bone tissue engineering are scaffolds. The use of scaffolds in bone grafts can be a solution to help the bone regeneration process (Bariyah et al., 2016) [8].

Bone tissue reconstruction can be performed with tissue engineering techniques, such as scaffolds that provide support as an extracellular matrix, allowing cells to proliferate and differentiate (Kamadjaja, 2021) [9]. Many studies have confirmed the feasibility of bone tissue reconstruction through tissue engineering (He et al., 2015) [10]. Tissue engineering is an interdisciplinary field of science that applies principles of engineering and biological sciences to develop biological substitutes that can restore, maintain, or improve tissue function (Carfì Pavia et al., 2018) [11]. Bone regeneration through bone tissue engineering involves several components such as stem cells, growth factors, cytokines, hormones, differentiation factors, and extracellular matrix (ECM) to accelerate healing (Rather et al., 2019; Qasim et al., 2020) [12]. Tissue engineering consists of three general components: scaffolds for transplantation and cell support, cells that can form a functional matrix, and bioactive factors that support and regulate cell activity (Doblado et al., 2021) [13].

Scaffolds are temporary supports that have a three-dimensional shape with a porous structure and have biophysical and biochemical conditions suitable for cell proliferation to remain integrated in the host tissue without the risk of rejection, accelerate angiogenesis in synergistic actions, and support mesenchymal cell differentiation (Ponciano et al., 2021) [14]. Research conducted by Danilchenko et al. (2011) [15] showed porous scaffolds with a combination of chitosan-hydroxyapatite (CS-HA) materials have shown good osteoconductive properties. CS-HA scaffolds result in increased biomineralization and protein adsorption capacity of biomimetic scaffolds used for bone tissue engineering (Nga et al., 2020) [16]. Chitosan (CS) is a cationic polysaccharide consisting of D-glucosamine and N-acetyl-D-glucosamine residues that have received great attention in bone tissue engineering (Shakir et al., 2018) [17]. CS has non-toxic, biocompatible, mucoadhesive, and biodegradable characteristics making it suitable for tissue engineering applications (Pitrolino et al., 2022) [18]. The hydrophilic surface, biocompatibility, and biodegradability of CS have a significant effect on enhancing cell adhesion, proliferation, migration, and differentiation (Brun et al., 2021) [19].

Hydroxyapatite (HA) is one of the most frequently used hydrated calcium phosphate biomaterials (gold standard) for bone regeneration applications because it has a composition and structure similar to bone and tooth minerals (Thariga et al., 2019) [20]. HA has excellent biocompatibility, bioactivity, and osteoconduction characteristics and can induce new bone adhesion and excellent bone integration (Sheikh et al., 2017; Thariga et al., 2019; Iaquinta et al., 2021; Zhang et al., 2021) [21] [22] [23]. The addition of HA to the chitosan structure can increase compressive strength, improve cell adhesion, enhance osteogenesis, reduce the swelling percentage of the scaffold, and maintain an appropriate degradation rate of the scaffold (Nga et al., 2020; Pitrolino et al., 2022).

The size of scaffold-forming particles can affect porosity and pore size (Prieto et al., 2015) [24]. The pore structure of the scaffold is an important factor in the process of angiogenesis and bone regeneration. The effective macro pore size limit for the bone regeneration process is 100 μm. When the pore size decreases cell viability, cell proliferation and differentiation are delayed, so that fibrous tissue is formed more than bone (Hayashi et al., 2020) [25]. Pore structure such as pore size, porosity, and pore interconnectivity can affect the osteogenic properties of the scaffold. Pore structure plays an important role in in vivo osteogenesis for bone reconstruction materials. The porosity of the scaffold must be high enough to support cell migration and nutrient/metabolite exchange. Interconnected pores ensure good transport, which is beneficial for nutrient transportation as well as cell and bone tissue penetration (Lu et al., 2020) [26].

The use of scaffolds can be combined with live cells and/or biologically active molecules to induce bone tissue repair and regeneration. This technique is the gold standard for conventional grafting (Nga et al., 2020). Human Umbilical Cord Mesenchymal Stem Cells (hUCMSCs) are a new source of Mesenchymal Stem Cells (MSCs) obtained from the umbilical cord of newborns and are widely considered to be an alternative to stem cell therapy. hUCMSCs are proven to increase osteoblast differentiation. This is supported by research conducted by Hendrijantini et al. (2018) [27] which showed that there was an increase in the number of osteoblasts, TGF-β1, and RUNX2 expression in osteoporotic mandibular bone. In addition, hUCMSCs have many advantages over other MSCs due to their easy retrieval process, minimally invasive, easier isolation process, large cell content, low immunogenicity that avoids immune rejection, and fewer bioethical issues (Hendrijantini et al., 2018; Yin et al., 2019) [28].

The use of umbilical cords that can be stored as frozen stock in liquid nitrogen will facilitate storage for many years (Borys-Wójcik et al., 2019) [29]. Frozen stock is hUCMSCs that have passed the freezing procedure by being stored in liquid nitrogen in a medium containing a cryoprotective agent. The cryoprotective agent serves to reduce the freezing point of the medium and slow down the freezing process to reduce the risk of ice crystal formation that can result in cell

death. Based on research conducted by Choudhury et al. (2013) [30] showed no significant difference in cell population from fresh and frozen hUCMSCs. This shows that cryopreservation allows the procedure of storing or banking hUCMSCs. Gong et al. (2012) [31] in their study mentioned that the banking of MSCs is necessary for future regenerative research and therapy, thus the need for a standardized process for the banking system of hUCMSCs. Research conducted by Balci & Can (2013) [32], showed that the cryopreserved hUCMSC method is easier to use and more efficient to produce high cell survival. Cryopreserved hUCMSCs produce more abundant MSC products, save costs and time, and have the same potential as fresh stock (Horie et al., 2021) [33].

The process of bone remodeling can be seen by examining bone remodeling biomarkers to describe the overall activity of osteoblast and osteoclast cells in bone (Huldani, 2012) [34]. 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 (Mahmudati, 2016) [35]. Increased serum levels of osteocalcin are associated with increased bone mineral density (Rathore et al., 2016) [36].

Osteocalcin or bone protein γ-carboxyglutamic acid (Gla) is a matrix protein that regulates bone mineralization and regulates the biological processes of various organs including bone (Wang et al., 2021) [37]. Osteocalcin activity is known to increase along with the process of osteoblast differentiation. Osteocalcin is produced during bone formation at the end of the mineralization process (Bailey et al., 2017) [38]. The process of alveolar bone remodeling begins to occur marked by the formation of osteoblast cells and bone spicules on day 14. Osteoblast cells and bone spicules are found more on day 21. On day 28, complete calcification of bone occurs (Puspita et al., 2022) [39]. The remodeling phase lasts from day 21 to about 1 year. However, osteoblast activity has started since day 14 with the formation of osteoblasts at the edge of the bone (Primadina, 2019) [40].

Research conducted by Kamadjaja (2021) on bone remodeling using CS-HA scaffolds seeded by hAMSCs, shows that osteocalcin secretion will increase when osteoblasts have turned into osteocytes. In that study, hUCMSCs have not been used as inducing cells for CS-HA scaffold on osteocalcin secretion. Therefore, this study will observe the effect of CS-HA scaffold on osteocalcin secretion by seeding hUCMSCs.

2. Literature Review

2.1. Osteocalcin

Osteocalcin (OC) is also called γ-carboxyglutamic acid (Gla) bone protein or bone gla protein (BGP). OC is an important component of the bone extracellular matrix in the form of non-collagenous proteins that are most commonly found in bone (Kini & Nandeesh, 2012; Li et al., 2016; Komori, 2020) [41] [42] [43]. OC is a matrix protein that regulates bone mineralization and regulates the biological processes of various organs including bone, brain, liver, pancreas, testis, muscle, parasympathetic nervous system, and adipose tissue (Wang et al., 2021). OC is abundantly produced by osteoblasts while smaller amounts are produced by odontoblasts and hypertrophic chondrocytes.

OC has been used as a serum marker of osteoblastic bone formation and affects the bone matrix to regulate mineralization (Zoch et al., 2016) [44]. Osteocalcin levels can be measured by electrochemiluminescence immunoassay (ELISA) (Atalay et al., 2012) [45]. Osteocalcin has a high affinity for calcium and undergoes conformational changes that bind to γ-carboxyglutamic acid (Gla) residues, causing the absorption of hydroxyapatite in the bone matrix. In this process, a mechanism occurs that allows osteocalcin to initiate the formation of hydroxyapatite crystals to mineralize bone (Jagtap et al., 2011; Zoch et al., 2016) [46].

2.2. Osteopontin

Osteopontin (OPN) is a phosphorylated glycoprotein that plays an important role in the pathological function and physiological response of the body (Hoo and Gatam, 2012) [47]. OPN is a non-collagen matrix protein component that facilitates matrix cell interactions (Hoo and Gatam, 2012: Aditiyono et al., 2018) [48]. OPN belongs to the small integrin binding ligand N-glycosylated (SIBLINGs) group, which is a group of 5 integrins related to glycophosphoproteins and is found in odontoblasts, osteoblasts, and osteocytes (Aditiyono et al., 2018; Boskey et al., 2012) [49]. OPN plays an important role in regulating vascular classification and regulating bone remodeling (Mohamed et al., 2021) [50].

In bone, osteopontin is produced by osteblasts during the pre-mineralization stage and the final stage of osteoblast maturation. Osteopontin plays an important role in bone tissue regeneration, such as cell adhesion, remodeling, and osseointegration in biomaterials or tissues that are generally found around mineralized tissues. Osteopontin plays a special role in the formation of osteoblasts in the early stages of bone formation. Osteopontin can also interact with

several cell surface receptors and plays an active role in many physiological processes such as wound healing, bone turnover, inflammation and angiogenesis. In addition, OPN dose-dependently increases MSC proliferation (Carvalho et al., 2021) [51].

2.3. Scaffold Chitosan-Hydroxyapatite (CS-HA)

Chitosan-hydroxyapatite (CS-HA) combination scaffolds are currently of interest in bone tissue engineering because they have elastic characteristics and compression resistance by chitosan and osteoinduction ability by hydroxyapatite. CS-HA scaffolds are biocompatible and induce bone stability (Rodríguez-Vázquez & Ramos-Zúñiga, 2020) [52]. Research conducted by Xianmiao et al. (2009) [53] showed that the surface roughness and micropores of the scaffold increased as the nHA content increased, making it suitable for cell adhesion, crawling, and growth. Cell culture and MTT assays showed that nHA and its content can affect cell proliferation. The CS-nHA scaffold has no negative effects on cell morphology, viability, and proliferation, and has good biocompatibility. This study makes the CS-nHA scaffold a prospective biodegradable bone regeneration guide for future applications. In addition, the study of Zhang et al. (2013) [54] showed that the CS-HA scaffold has a radius-like, layered, and porous structure. SEM examination of the CS-HA scaffold after seven days of cell culture showed that the cells grew, adhered, and spread well. CS-HA scaffolds have good in vitro biocompatibility. It can be seen that CS-HA scaffolds have higher cell proliferation ability than pure CS scaffolds.

2.4. Human Umbilical Cord Mesenchymal Stem Cells (hUCMSCs)

MSCs derived from the baby's umbilical cord are also called human Umbilical Cord Mesenchymal Stem Cells (hUCMSCs) which have multipotent properties and are starting to be considered as an MSCs option (Dahlan et al., 2020) [55]. hUCMSCs are obtained directly from Wharton's jelly from the human umbilical cord which is also called Wharton's jelly mesenchymal stem cells (WJ-MSCs) (Li et al., 2017) [56]. hUCMSCs are one of the media for application in the regeneration of periapical bone, pulp, and alveolar bone (Prasetyo et al., 2021) [57].

HUCMSCs have the advantage of being easy to obtain because they are biological waste that is always discarded or unused so ethically there will be no obstacles to use (Dahlan et al., 2020). In addition, hUCMSCs have simpler characteristics, a large number of availability, are non-invasive in terms of collection, have high proliferation potential and high differentiation, lack of morbidity during the immunosuppressive retrieval process, have a high level of immunocompatibility, and are easily reproduced (Hendrijantini & Hartono, 2019; Kuntjoro et al., 2020) [58] [59]. Due to their bioactive advantages, hUCMSCs are likely to become a promising new approach for tissue repair and regeneration (Yin et al., 2019).

2.5. Mesenchymal Stem Cell (MSCs)

Mesenchymal stem cells (MSCs) have attracted attention recently as they have great therapeutic potential (Li et al., 2017). MSCs are the most frequently used cells in tissue engineering. MSCs can differentiate into other cells in the process of tissue healing. MSCs can differentiate into collagen, osteoblasts, chondrocytes, and so on. The right stimulus and environment are very influential in the process of MSC differentiation into more specific cells (Utomo & Widiastana, 2019) [60]. The emergence of MSCs-based therapy as a clinical therapy is an innovation in the treatment of various diseases related to inflammation and tissue damage to regenerate and repair (DiMarino et al., 2013) [61]. MSCs have differentiation capabilities that can perform self-renewal and multi-directional (Yin et al., 2019). MSCs are multipotent progenitor cells that can be obtained from various tissues in the body, including adult bone marrow, adipose tissue, skin, umbilical cord, and placenta. MSCs can migrate to the defect site and help the bone regeneration process (Jimi et al., 2012) [62]. Examples of MSCs include the amniotic membrane, chorion plate, parietal decidua, and umbilical cord (Hendrijantini et al., 2019) [63].

3. Methodology

This study is an analytical laboratory experimental research (true experimental laboratories). The sample used was CS-HA scaffold which was seeded with cryopreserved hUCMSCs then osteocalcin secretion was observed. Osteocalcin secretion test data obtained through optical density ELISA readings were 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.

4. Results

4.1. Immunocytochemistry Examination Results

Immunocytochemistry examination showed that hUCMSCs have the characteristics of MSCs markers, namely spindle-shaped cells, non-fluorescent cells that express negatively on CD45 marker (figure 1. A and B), and fluorescent cells that express positively on CD90 marker (figure 1. C and D).

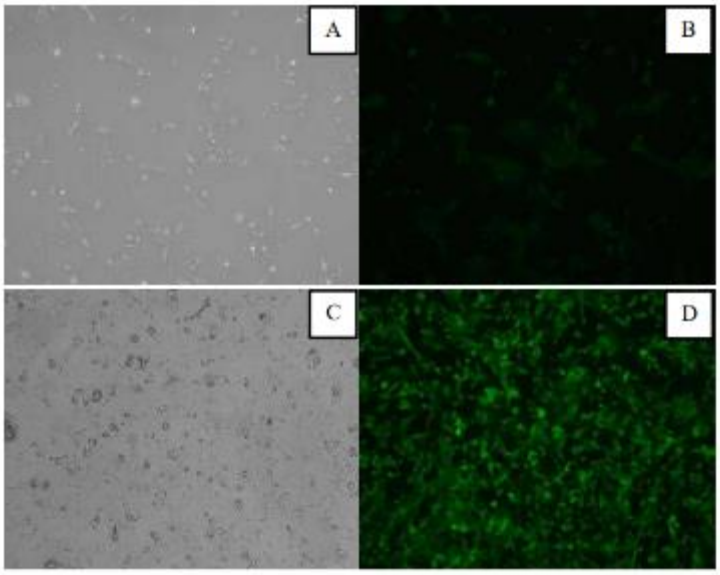

Figure 1 Immunocytochemistry test examination of (A) CD45 contrast phase, (B) CD45 fluorescent phase, (C) CD90 contrast phase, and (D) CD90 fluorescent phase.

4.2. Scanning Electron Microscope (SEM) Inspection Results

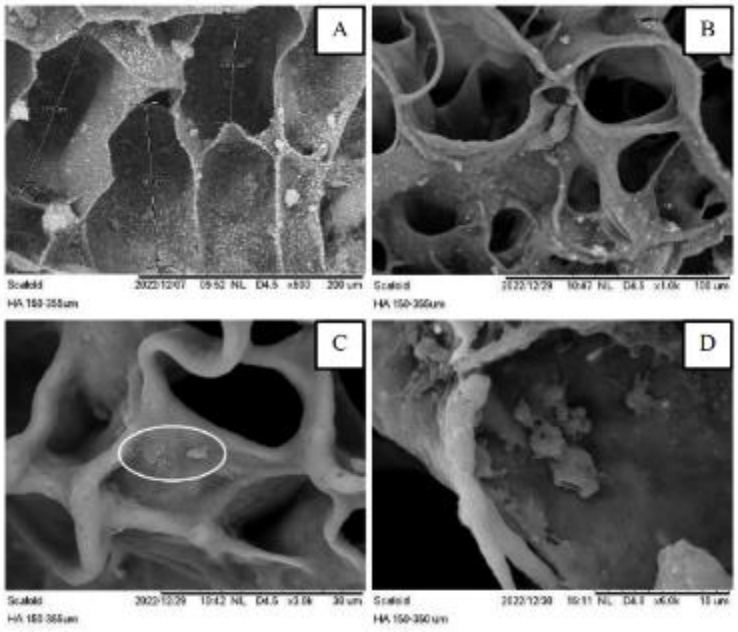

Figure 2 SEM examination of CS-HA scaffold without hUCMSCs induction with a magnification of (A) 500x, (B) 1000x. SEM examination of CS-HA scaffold induced by hUCMSCs 3x24 hours with magnification (C) 3000x, (D) 6000x

SEM examination was conducted on the CS-HA scaffold that was not induced by hUCMSCs to evaluate the pore size of the scaffold. In the SEM examination with 500x magnification (Figure b. A), the pore size was found to be 99.5 µm, 147 µm, and 156 µm.

SEM examination of CS-HA scaffold induced by hUCMSCs for 3x24 hours to determine the morphological details of cells attached to the scaffold. SEM examination with 6000x magnification (Figure b. D) showed the attachment of cells to the scaffold wall.

4.3. Total Osteocalcin Secretion on Day 14 and 2

The amount of osteocalcin secretion from the differentiated cells on days 14 and 21 was seen through ELISA examination. The results will be based on absorbance readings, i.e. optical density (OD) values in each treatment group presented in table 1.

Table 1 Research Data

Day - 14			**Day - 21**		
KP1	KN1	P1	KP2	KN2	P2
38.7619	30.85714	34.95238	46.38095	40.66667	39.71429
37.33333	31.09524	37.80952	46.85714	43.52381	40.66667

Based on the research data in Table C. 1, descriptive analysis can be carried out as in Appendix 4 and presented in Table 2. in the form of mean values and standard deviations in each treatment group on days 14 and 21.

Table 2 The amount of osteocalcin secretion on days 14 and 21.

Day -	**Group**	**Average ± SD**
14	KP1	40.67 ± 3.99
	KN1	33.20 ± 2.60
	P1	36.02 ± 1.25
21	KP2	48.52 ± 2.41
	KN2	42.57 ± 1.35
	P1	42.33 ± 2.50

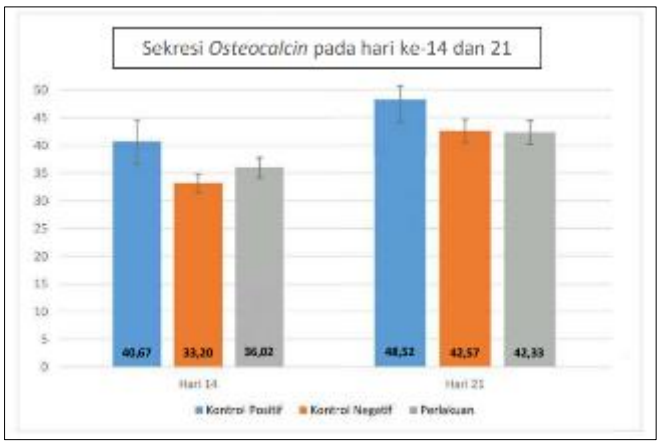

Figure 3 The amount of osteocalcin secretion on days 14 and 21

Based on Table 2 and Figure 3, it can be seen that the average amount of osteocalcin secretion on day 21 of the three groups has a higher value than the amount of osteocalcin secretion on day 14 of the three groups. The osteocalcin

secretion in the positive control group had a higher average value than the negative control group and the treatment group both on day 14 and day 21. On day 14, the mean value of the amount of osteocalcin secretion in the positive control group had the highest value (40.67 ± 3.99) followed by the treatment group (36.02 ± 1.25) and then the negative control group (33.20 ± 2.60). On the 21st day, the average value of the amount of osteocalcin secretion in the positive control group had the highest value (48.52 ± 2.41) followed by the negative control group (42.57 ± 1.35) and the treatment group (42.33 ± 2.50). Thus, osteocalcin secretion in the positive control group on day 21 had the highest mean value of all treatment groups, which was 45.52 ± 2.41.

4.4. Differences in the Amount of Osteocalcin Secretion on Days 14 and 21

Analysis of differences in the mean amount of osteocalcin secretion on days 14 and 21 in all groups will be carried out using the One-way ANOVA test to determine whether there are differences in the amount of osteocalcin secretion in these groups with the condition that the data of all groups have homogeneous and normally distributed data variations.

4.5. Normality Test of Total Osteocalcin Secretion on Days 14 and 21

In this study, a normality test will be carried out to determine that the research data on the amount of osteocalcin secretion on days 14 and 21 from all groups are normally distributed. The normality test will be measured using the probability value in the Shapiro-Wilk test. In this test, the statistical hypothesis used is as follows:

H_0: The research data is normally distributed

H_1: The research data is not normally distributed

The test criteria for the statistical test used are the significance value, if the significance value is smaller than 0.05 then H_0 is accepted or the data is normally distributed, and vice versa. The following are the results of the normality test using the Shapiro-Wilk test:

Table 3 Normality test for the amount of osteocalcin secretion on days 14 and 21

Group	**Shapiro-Wilk**		
	Statistic	df	*p*
KP1	0.875	4	0.319
KN1	0.832	4	0.173
P1	0.887	4	0.369
KP2	0.915	4	0.507
KN2	0.827	4	0.161
P2	0.811	4	0.123

Based on the results of the normality test in Table 3, it can be seen that all groups have a p value> 0.05 and H_0 is accepted so that the data on the amount of osteocalcin secretion on days 14 and 21 are normally distributed and can qualify for the One-way ANOVA test.

4.6. Homogeneity Test of Total Osteocalcin Secretion on Days 14 and 21

In this study, a homogeneity test will be carried out to determine that the research data on the amount of osteocalcin secretion on days 14 and 21 from all groups have homogeneous data variations. Data homogeneity will be measured using Levene's Test. In this test, the statistical hypothesis used is as follows:

H_0: The research data varies homogeneously

H_1: Research data varies heterogeneous

The test criteria for the statistical test used is the significance value, if the significance value is smaller than 0.05 then H_0 is rejected or the data varies homogeneously, and vice versa. The following are the results of the homogeneity test using Levene's Test:

Table 4 Homogeneity test of the amount of osteocalcin secretion on days 14 and 21.

Group	**Levene's Test for Equality of Variances**	
	F	*p*
Osteocalcin secretion on days 14 and 21	1.727	0.179

Based on the results of the homogeneity test in Table 4, it can be seen that all groups have a p value> 0.05 and H_0 is accepted so that the data on the amount of osteocalcin secretion on days 14 and 21 have homogeneous variations and can qualify for the One-way ANOVA test.

4.7. ANOVA test for differences in osteocalcin secretion on days 14 and 21

Based on the results of the previous analysis, all data have met the requirements of homogeneity and normality of data so the measurement of the difference in the mean amount of osteocalcin secretion on days 14 and 21 will be analyzed using the One-way ANOVA test to determine if there is a significant difference between the six groups. In this test, the statistical hypothesis used is as follows:

H_0: There is no difference in the average osteocalcin secretion in all groups (there is no effect of the combination of treatments given on the value of osteocalcin secretion)

H_1: There is a difference in the average osteocalcin secretion in all groups (there is an effect of the combination of treatments given on the value of osteocalcin secretion).

The test criteria for the statistical test used are the significance value, if the significance value is smaller than 0.05 then H_0 is rejected or there is a difference in the average OD value in all groups, and vice versa. The following are the results of the comparison using the One-way ANOVA test:

Table 5 ANOVA test for differences in osteocalcin secretion on days 14 and 21.

	Sum of Square	**df**	**Mean Square**	**F**	$\mathbf{F_{(5,18)}}$	***p***
Between Group	581.342	5	116.268	18.301	2.77	0.000
Within Group	114.353	18	6.353			
Total	695.695	23				

The results of the One-way ANOVA test in Table 5 show that the difference in the amount of osteocalcin secretion on days 14 and 21 has a p-value (0.000) which is smaller than 0.05 ($p < 0.05$) and H_0 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 value of F (18.301) > F5.18 (2.77) so that H_0 is rejected, thus it can be concluded that there is a significant difference between the amount of osteocalcin secretion on days 14 and 21. Significant differences can then be analyzed again with the Post Hoc Test using Tukey-HSD.

4.8. Post Hoc Test for Differences in Osteocalcin Secretion on Days 14 and 21

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

Table 6 Tukey-HSD Test Results

	KP 1	KN 1	P 1	KP2	KN2	P2
KP 1		0.006*	0.147	0.004*	0.887	0.932
KN 1	0.006*		0.619	0.000*	0.001*	0.001*
P 1	0.147	0.619		0.000*	0.018*	0.024*
KP 2	0.004*	0.000*	0.000*		0.036*	0.028*
KN 2	0.887	0.001*	0.018*	0.036*		1.000
P 2	0.932	0.001*	0.024*	0.028*	1.000	

Notes: (*) indicates significant difference ($p < 0.05$)

Based on the Tukey-HSD test results, it can be seen that the mean value of osteocalcin secretion in the positive control group on day 14 has a significant difference with the positive control group on day 21 and the negative control group on day 14 but is not significantly different from the treatment group on day 14, the treatment group on day 21, and the negative control group on day 21. The average value of osteocalcin secretion in the positive control group on day 21 has a significant difference in all groups. The 21st-day treatment group had a significant difference from the 14th-day treatment group, the 14th-day negative control group, and the 21st-day positive control group.

5. Discussion

Bone tissue reconstruction can be performed with tissue engineering techniques. Currently, biomaterials that are widely developed in bone tissue engineering are scaffolds. The scaffold functions as an extracellular matrix that allows cells to proliferate and differentiate (Kamadjaja et al., 2016) [64]. Scaffolds that will act as carriers of bioactive agents to improve bone cell function and induce new bone formation (Jahan et al., 2020) [65]. Research conducted by Danilchenko et al. (2011) showed porous scaffolds with a combination of chitosan-hydroxyapatite (CS-HA) materials have shown good osteoconductive properties.

The use of scaffolds can be combined with live cells and/or biologically active molecules to induce bone tissue repair and regeneration. This technique is the gold standard for conventional grafting (Nga et al., 2020). hUCMSCs can be considered as an alternative treatment for stem cell therapy and have been shown to increase osteoblast differentiation. This is supported by in vitro research conducted by Kangari et al. (2020) [66] which proved that hUCMSCs can promote osteoblast formation and prevent osteoclast cellular activity.

This study uses frozen (cryopreserved) hUCMSCs that have passed the freezing procedure by being stored in liquid nitrogen in a DMSO medium containing cryoprotective agents. Based on research conducted by Choudhery et al. (2013) showed no significant difference in cell population from fresh and frozen hUCMSCs. Research conducted by Balci & Can (2013), showed the cryopreserved hUCMSC method is easier to use and more efficient to produce high cell survival. Cryopreserved hUCMSCs produce more abundant MSC products, save costs and time, and have the same potential as fresh stock (Horie et al., 2021). hUCMSCs used have been confirmed to be MSCs by immunocytochemistry examination which shows positive expression of CD90 and negative expression of CD45.

Based on the results of the study, osteocalcin secretion obtained from CS-HA scaffold-induced hUCMSCs with osteogenic medium on days 14 and 21 was found to be significantly different ($p=0.024$) based on the Tukey-HSD test. The same result was also obtained from CS-HA scaffolds-induced hUCMSCs with α-MEM medium on days 14 and 21, which showed a significant difference ($p=0.004$) and hUCMSCs cultured in osteogenic medium on days 14 and 21 showed a significant difference ($p=0.001$). Thus, it can be concluded that on day 21 there is an increase in osteogenic characterized by an increase in osteocalcin secretion. This is in line with the research of Soriente et al. (2018) [67] which explained that the peak of osteocalcin secretion occurred on day 21.

The process of bone remodeling can be seen by examining bone remodeling biomarkers to describe the overall activity of osteoblast and osteoclast cells in bone (Huldani, 2012). Osteocalcin levels are related to the process of bone formation so it can be used as a marker or biomarker of bone formation (Rubert & de la Piedra, 2021) [68]. Serum levels of total osteocalcin, Gla osteocalcin, and Glu osteocalcin will increase when there is an increase in bone formation (Komori, 2020).

Osteocalcin is the most abundant non-collagenous bone matrix protein expressed by osteoblasts. Osteocalcin plays an important role in regulating the mineralization process during osteogenic differentiation of MSCs (Tsao et al., 2017) [69]. Osteocalcin activity is known to increase along with the osteoblast differentiation process. Osteocalcin is produced during bone formation at the end of the mineralization process (Bailey et al., 2017). Research conducted by Ardeshirylajimi et al. (2015) [70] showed an increase in the expression of osteogenic markers (osteocalcin, RUNX2, ALP, osteonectin) over a 21-day culture period in a porous freeze-dried chitosan scaffold seeded with ADMSCs.

In the positive control group and treatment group, CS-HA scaffold and stem cells in the form of hUCMSCs were added. From Figure A, the average amount of osteocalcin secretion in the negative control group tends to be lower than in the positive control group and the treatment group. In addition, table h shows that the negative control group on day 14 has significant differences in almost all groups, except for the treatment group on day 14 ($p=0.619$). The negative control group on day 21 had a significant difference with the treatment group on day 14 ($p=0.018$) and the positive control group on day 21 ($p=0.036$).

MSCs can differentiate into osteoprogenitors and osteoblasts and form a calcified bone matrix (Tsao et al., 2017). Research conducted by Zhang et al. (2020) [71] showed that hUCMSCs can differentiate into odontoblast-like cells and functional endothelial cells. In addition, the results of the qRT-PCR examination showed that the expression level of osteocalcin was significantly higher in the cell-seeded scaffold group compared to the control group. CT scan, radiography, immunohistochemistry, biomechanical, histopathology, and histomorphometric examinations revealed a significantly higher bone regeneration process characterized by the formation of new bone tissue in the MSCs-seeded scaffold group than the group that was not given MSCs (Oryan et al., 2020) [72].

Research conducted by Soriente et al. (2022) [73] showed that CS-HA scaffolds can support the proliferation and osteogenic differentiation of hMSCs thanks to the important chemical properties of chitosan and the bioactive/osteoconductive properties of hydroxyapatite. Rodríguez-Vázquez & Ramos-Zúñiga (2020) revealed that the potential of the CS-HA scaffold can induce bone regeneration to be dense and well-structured. According to Thariga et al. (2019), HA/CS/Gelatin scaffolds have cell-matrix interactions with potential wound-healing capabilities, increasing the effectiveness of the approach to tissue engineering applications. In addition, in vitro biological evaluation conducted by Kim et al. (2013) [74] showed that hMSCs cultured on CS-HA scaffolds showed increased proliferation compared to hMSCs cultured on pure CS scaffolds.

Based on the results of the study, the average amount of osteocalcin secretion in the positive control group on day 21 has the highest amount and has a significant difference 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 hUCMSCs cultured in α-MEM medium. According to Grossner et al. (2022) [75], osteogenic differentiation of MSCs is becoming a standard procedure in modern bone tissue engineering. One of the basal cell culture media in tissue engineering cell culture is α-MEM. α-MEM media is suitable for osteogenic differentiation because it contains non-essential amino acids, Vitamin B12, biotin, and ascorbic acid. Research conducted by Grossner et al. (2022) showed that strong osteogenic differentiation can be achieved using basal cell culture media such as DMEM and α-MEM. Differentiation of MSCs using α-MEM will lead to higher acid phosphatase activity which is also a good marker for osteogenic response.

The pore structure of the scaffold is an important factor in the process of angiogenesis and bone regeneration. SEM examination of the CS-HA scaffold that was not induced with hUCMSCs at 500x magnification (Figure 2. A) revealed pore sizes of 99.5 μm, 147 μm, and 156 μm. Hayashi et al. (2020) revealed that the effective macro pore size limit for the bone regeneration process is 100 μm. When the pore size is <100 μm, cell distribution and angiogenesis throughout the scaffold are reduced. From the measurement results, it was found that the pore size in the CS-HA scaffold with HA particle size of 150-355 μm met the requirements.

SEM examination of the CS-HA scaffold induced by hUCMSCs for 3x24 hours with 6000x magnification (Figure 2. D) showed the presence of cell attachment to the scaffold wall. This is in line with the research of Chatzipetros et al. (2021) [76] which shows that porous scaffolds consisting of nano Hydroxyapatite (nHA) and chitosan have high cell attachment, increased pre-osteoblast response, cell proliferation, and well-distributed cell distribution in the scaffold biomaterial structure. In addition, research conducted by Pitrolino et al. (2022) explained that MSC attachment and proliferation were supported by chitosan scaffolds enhanced by the addition of nHA. SEM examination on day 2 after seeding MSCs showed sheets of cells attached to the CS-nHA scaffold. El-Bassyouni et al. (2020) [77] revealed that cell proliferation ability was established after 3 and 7 days. SEM images showed that stem cells and epithelial cells attached to the scaffold obtained full integration between cells and scaffold material.

Pore structures such as pore size, porosity, and pore interconnectivity can affect the osteogenic properties of the scaffold. The porosity of the scaffold should be high enough to support cell migration and nutrient/metabolite exchange (Lu et al., 2020). The porous surface of the scaffold serves to facilitate cell attachment, growth, proliferation, and differentiation by forming an extracellular matrix (Herda & Puspitasari, 2016) [78]. The porosity contained in the scaffold becomes a space for cells to attach and grow into new bone tissue (Bariyah et al., 2016).

6. Conclusion

From this study, the results were obtained by the hypothesis, namely osteocalcin secretion on CS-HA scaffold with hUCMSCs induction in vitro showed the process of bone regeneration. This was shown by the average amount of osteocalcin secretion produced by the induction of hUCMSCs on CS-HA scaffolds in α-MEM medium on day 14 which increased on day 21 and had the highest amount of osteocalcin secretion of all groups. The significant difference in the mean amount of osteocalcin secretion on days 14 and 21 indicates osteogenic differentiation.

Further research needs to be done using other osteogenic biomarkers that have not been studied in hUCMSCs to provide more insight into the process of bone regeneration.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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