Increased Expression of Runx2 and Osteocalcin in Freeze Dried Bovine Bone Scaffold-Secretome Mesenchymal Stem Cell (In Vitro Laboratory Experimental Research on MC3T3-E1 Pre-Osteoblast Cell Culture)

Danang Priyo Utomo[1,2], David Buntoro Kamadjaja[3]*, Ni Putu Mira Sumarta[3], Coen Pramono[3], Andra Rizqiawan[3], Andang Miatmoko[4], Eryk Hendrianto[5], Mohammad Zeshaan Rahman[3,6]

1.Magister Program of Clinical Medicine, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.
2.Resident of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
3.Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga,, Surabaya, Indonesia.
4.Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia.
5.Stem Cell Research and Development Center, Universitas Airlangga, Surabaya, Indonesia.
6.Associate Professor Department of Oral and Maxillofacial Surgery Pioneer Dental College and Hospital, Dhaka, Bangladesh.

Abstract

Reconstruction of maxillofacial defect still remains a challenge for oral and maxillofacial surgeons. Autograft, which has been the gold standard, has limitations and donor site morbidity. Tissue engineering combining cells, scaffold, and growth factors is expected to produce ideal bone without morbidity, with Human umbilical cord mesenchymal stem cells (HUCMSC) and bovine scaffold are promising topics. HUCMSC therapy is influenced by biomolecules including cytokines, growth factors, and microRNAs, known as secretome. This study aims to analyze the osteoinduction potential of the HUCMSC secretome in the FDBBX scaffold through the expression of Runt Related Transcription Factor-2 (RUNX2) and Osteocalcin (OCN) markers.

Objectives to analyze the increased expression of RUNX2 and OCN of MC3T3-E1 preosteoblast cells that were seeded into FDBBX scaffold after application of the HUCMSC secretome compared to scaffold without secretome.

The study was an in vitro laboratory experiment. The first group was FDBBX scaffold after HUCMSC secretome application and the second group was FDBBX without secretome. The analysis of the RUNX2 and OCN expression of MC3T3-E1 pre-osteoblast cells that attached to the scaffold was conducted on days 7, 14 and 21 using RT-PCR method. Independent T test was conducted as a Statistical test to analyze the differences between groups, and One way ANOVA test was conducted to analyze the difference between time. The difference analysis was considered significant when p<0,05.

The Independent T test result showed that the expression of RUNX2 and OCN in FDBBX scaffold after HUCMSC secretome application was significantly higher than scaffold without secretome (P=0,039; P=0,025).

The expression of RUNX2 and OCN of MC3T3-E1 pre-osteoblast cells in the FDBBX scaffold after HUCMSC secretome application was higher than the scaffold without secretome application.

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Introduction

The management of bone defects in the maxillofacial region due to tumor excision, trauma, infection, iatrogenic, or radiation therapy remains a challenge for oral surgeons.[1] Autograft is the gold standard therapy because it includes the osteoinductive, osteoconductive, and osteogenic capacities but it has disadvantage of the limited number of grafts and morbidity of the donor site. Non-autogenous bone grafts such as allograft, xenograft, and alloplast are another option to be considered. The bone graft is available in the form of mineralized, demineralized, fresh, frozen, or freeze-dried. However, grafts that originally do not come from the individual itself have higher risk of disease

*Corresponding author:
David Buntoro Kamadjaja
Department of Oral and Maxillofacial Surgery,
Faculty of Dental Medicine, Universitas Airlangga.
St. Prof. Dr. Moestopo No. 47, Surabaya, 60132, Indonesia.
E-mail: david-b-k@fkg.unair.ac.id

transmission and rejection reactions from host tissues.[2,3] Tissue engineering is a new alternative which uses combination of cells, scaffold, and growth factors and is expected to produce ideal new bone growth without donor morbidity. The recent interest of bone tissue engineering is to create biomaterials that have ideal mechanical and biological properties.[4,5]

The success of bone regeneration through tissue engineering concept is highly dependent on osteoblast differentiation. The maturation of pre-osteoblast to mature osteoblast and finally osteocytes is always followed by the expression of specific markers such as Runt Related Transcription Factor-2, Osterix, Bone Sialoprotein, Osteopontin and Osteocalcin. RUNX2 is a master regulator of osteoblast maturation and most highly expressed during the early phase.[6,7] Osteocalcin is a Ca^{2+} ion binder, hydroxyapatite, and collagen which plays a role in hydroxyapatite deposition and expressed in intermediate and late phases of the osteoblast maturation.[8]

Bovine hydroxyapatite (BHA) as scaffold has been used as an bone graft material for the reconstruction of bone defects. BHA is affordable and has osteoconductive capacity that functions as a mechanical support and a three-dimensional framework for defects, but all the main organic components such as TGF-β and BMP-2 are extracted.[7] Furthermore, a new scaffold alternative was developed using freeze dry method. Freeze-dried bovine bone xenograft (FDBBX) is more ideal to be used as scaffold because it contains both organic and inorganic materials.[9,10] Growth Factor is an essential part in bone regeneration. The secretome of mesenchymal stem cell contains various biomolecules, including paracrine factors such as cytokines, growth factors, and microRNAs. Secretome regulates the process of osteoblast maturation and increase osteogenic markers through various signaling pathways such as TGF-β, Smad, MAPK, and WNT.[11,12] The use of the MSC secretome, or its components, has advantages over the implantation of the MSCs themselves: (i) signals can be bioengineered and scaled to specific dosages, and (ii) the non-living nature of the secretome enables it to be efficiently stored and transported.[13]

In this study, we analyse the increased expression of RUNX2 and OCN of MC3T3-E1 pre-osteoblast cells that were seeded into FDBBX scaffold after application of the HUCMSC secretome. The application of the HUCMSC secretome is expected to increase the osteoinduction ability of the FDBBX by increasing the expression of osteogenic markers.

Materials and methods

This research was an in vitro study to analyse the increasing RUNX2 and OCN expression of MC3T3-E1 pre-osteoblast cells that were seeded into Freeze Dried Bovine Bone Xenograft after application of Secretome Human Umbilical Cord Mesenchymal Stem Cells (research group) compared with Freeze-Dried Bovine Bone Xenograft only (control group). Each group respectively were observed for the expression of RUNX2 and OCN using RT-PCR with 3 times replication. In each group, observations were made at days 7, 14, and 21. The research was conducted in the laboratory of the Institute Tropical Disease Centre, Universitas Airlangga, Surabaya. Meanwhile, the production of FDBBX was in BATAN research bank.

Thawing and culture cell of MC3T3-E1 Preosteoblast Cell

The frozen MC3T3-E1 preosteoblast cell from *European Collection of Authenticated Cell Cultures* (ECACC) were thawed using waterbath 37^{O}C within 2 minutes. Cells were moved to a 15ml conical tube, then 4 ml α-modified minimal essential medium (α-MEM) from sigma aldrich was added and centrifugated at 100G within 7 minutes. The supernatant was removed and replaced with 1ml α-MEM. Finally, the cells were ready to be moved into the culture dish.

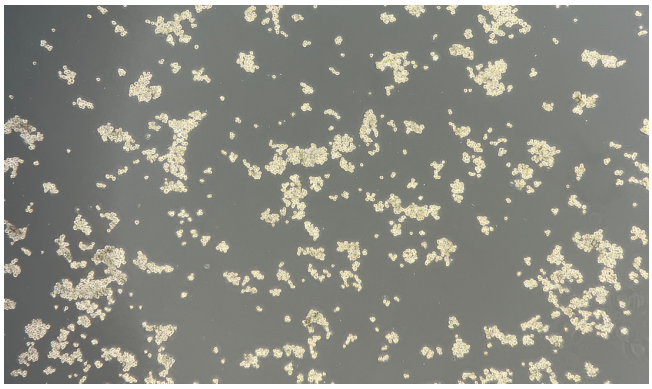

Figure 1. MC3T3-E1 preosteoblast cells after thawing process and hass reached 80% confluency. The cells were cultured and maintained at 37^{O}C 98 ^{O}C humidity, and 5% CO2.

Cells were cultured in alpha-MEM medium supplemented with 10% FBS, HEPES (PBS) 10mM, 100 units/ml penicillin, and 100 mg/ml streptomycin. The cells were maintained at 37^{O}C in a humidified atmosphere of 5% CO2 in the air and the medium was changed every 3 days. Cultures were sub-cultured until the cells reached 80% confluency and the eighth passage (figure 1).

Secretome and Medium Application in FDBBX Scaffold

Secretome HUCMSC was applied to each side of the scaffold using pipette in and out method on 6-well culture dish (40 µL each). In the control group, α-MEM medium was applied to the scaffold with the same volume and method as the research group. The scaffold was incubated for 24 hours with 37^{O}C, 5% CO_2, and 98% humidity.

Seeding Preosteoblast cells MC3T3-E1 in FDBBX Scaffold

MC3T3-E1 preosteoblast cells were seeded in well plates with 2x10^5 cells in each well. Plates are incubated in a standard culture condition (37^{O}C, 5% CO_2, and 98% humidity). The medium was changed using osteogenic medium (mesencult stem cells technologies) every 3 days using a pipet with in and out method.

Harvesting Preosteoblast Cell from Scaffold

After the scaffold had been incubated according to the observation time (7, 14, 21 day), the supernatant was discarded, leaving only the scaffold. The preosteoblast cell was then released from the scaffold by trypsination method using 500 triple express. Trypsination process ran for 10 minutes in the 37^{O}C, 5% CO_2, and 98% humidity condition and stopped using α-MEM. Scaffold centrifugation was at 2500 rpm for 5 minutes, then the supernatant was removed, leaving only the MC3T3-E1 cells.

RNA Extraction and RT-PCR

RNA extraction of MC3T3-E1 cells was carried out by ethanol application and washing method (Norgen Biotek Corp). The total RNA that had been extracted was used for the synthesis of complementary DNA. The composition of the master mix according to the Biorad PCR kit protocol consists of iscript reverse transcription (1 µL), iscript reaction mix (4 µL), nuclease free water (13 µL), and RNA research sample (2 µL). The cDNA synthesis protocol was carried out under the following conditions: The primary phase was annealed at 23^{O}C for 10 minutes, followed by reverse transcription at 42^{O}C for 60 minutes, and the termination phase at 94^{O}C for 10 minutes. cDNA was used as a template for RT-PCR to calculate the expression levels of the Runt Related Transcription Factor-2 (RUNX2) and Osteocalcin (OCN) genes and the expression results were obtained in the form of numbers. Primary and reverse sequences were based on the Biorad protocol (Table 1). All genes were normalized with lyceradehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene to assess the activity of RUNX2 and OCN expression (table 1).

Gene	*Forward primer sequence*	*Reverse primer sequence*
RUNX2	TGGAA TACGA ACTGG GATGA GAAG	GGAAC TGATA GGATG CTGAC GAAG
OCN	GGAGG GCAAT AAGGT AGTGA ACAG	GGCTT TAGGG CAGCA CAGGT C
GAPDH	GTGAG GCCGG TGCTG AGTAT GT	GCAGA AGGGG CGGAG ATGA

Table 1. Primary and reverse sequencer RUNX2, OCN and GAPDH.

Statistical Analysis

Statistical analysis was performed using IBM SPSS 25. Shapiro-wilk test was conducted for the normality and Levene's Test for homogeneity test. The Independent T-Test was carried out to evaluate the differences in RUNX2 and OCN expression between the control group and the treatment group. One-way ANOVA test was conducted to evaluate the differences among the groups based on the variation of observation time. The final test was Post Hoc (LSD).

Results

In this study, the average RUNX2 expression in secretome group was higher than control group on the 14th and 21st days of observation. On the 7th day of observation, RUNX2 expression in the control group was higher than the HUCMSC secretome group (see table 2).

The normality test using Kolmogorov-Smirnov and Shapiro Wilk test and the homogeneity test using Levene test showed that all data were normally distributed and homogeneous (P> 0.05). Statistical analysis using Independent T test showed that there is significant difference between two group on days 14 (P=0.039) (see table 2 and figure 2).

Meanwhile, on 7th and 21st day, there was no significant difference.

Days/Group		N	Mean	GAPDH	ΔΔCT	(Independent T-Test)
7	Control	3	35,07626979	19,49895223	14,57731755	0,258
	Secretome	3	33,11401304	20,41540782	12,69860522	
14	Control	3	34,18464661	17,9695371	15,21510951	0,039*
	Secretome	3	37,66206614	30,37525558	7,286810559	
21	Control	3	32,09562238	20,75259209	-53,84821447	0,190
	Secretome	3	34,48443212	22,79374758	11,69068453	

Table 2. The RT-PCR result of RUNX2 Expression.

*P<0,05 means there is significant difference.

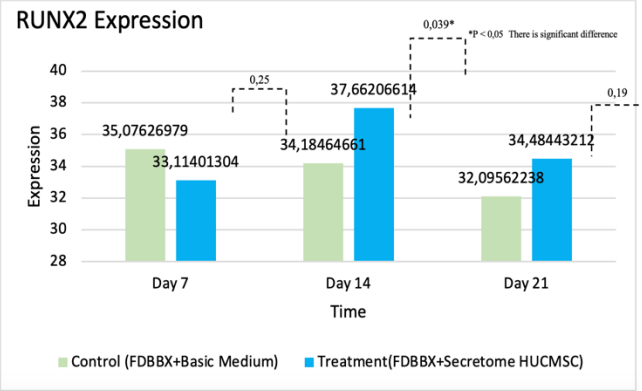

Figure 2. RT-PCR result for RUNX2. The average RUNX2 expression in secretome group was higher than control group on 14th and 21st days of observation. On 7th day, RUNX2 expression in control group was higher than in secretome group. Statistical analysis using Independent T test showed that there is a significant difference between two group on day 14.

The result showed that the highest RUNX2 expression occurred at the beginning of the observation. In control group, the highest expression of RUNX2 was on the 7th day, and then decreased on day 14th and 21st day. Meanwhile in the secretome group showed that the highest expression of RUNX2 occurred on the 14th day and decreased after 21st day (See Figure 3). Lowest result RUNX2 level show on the 7th day. Statistical analysis using the one-way ANOVA showed a significant difference in control group (P=0.034) but no significant difference in secretome group. Post hoc test show that there was significant difference between 7th and 21st day observation in control group. In secretome group, there was significant difference in 7th and 14st day observation (see table 3).

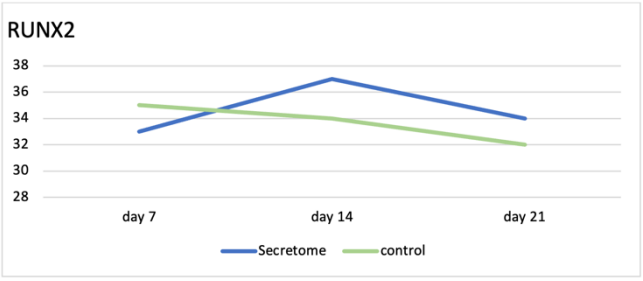

Figure 3. Activity of RUNX2 expression from day 7-21. In control group the highest expression of RUNX2 was on the 7th day, and then decreased on the 14th and 21st days. Meanwhile in the secretome group showed that the highest expression of RUNX2 occurred on the 14th day and decreased after 21st day.

Day/Day		Osteogenic Medium (Control)			*Secretome* HUCMSC		
		Sig. (Anova)	Sig. (LSD)	Std. Error	Sig. (Anova)	Sig. (LSD)	Std. Error
7	14	0,034	0,341	0,86325	0,100	0,043	1,77392
	21		0,014	0,86325		0,469	1,77392
14	7						
	21		0,052	0,86325		0,124	1,77392
21	7						
	14						

Table 3. RUNX2 Expression Statistical Analysis.

The average expression of osteocalcin in secretome group was higher than in control group on the 14th and 21st days. On the 7th day, both control and secretome group, no osteocalcin expression was observed (See table 4 and figure 2).

The normality test using Kolmogorov-Smirnov and Shapiro Wilk test and the homogeneity test using Levene test showed that all datas were normally distributed and homogeneous (P> 0.05). Statistical analysis using Independent T test showed that there is significant difference between two group on days 21 (P=0.025). Meanwhile, on 14th day, there was no significant difference (see table 4 and figure 4).

Day/Replication		N	Mean	GAPDH	ΔΔCT	Sig. (Independent T-Test)
7	Control	3	0	19,49895223	-21,49895223	0
	Secretome	3	0	20,41540782	-20,41540782	
14	Control	3	26,31068357	17,9695371	6,341146469	0,233
	Secretome	3	28,97621473	30,37525558	-1,399040858	
21	Control	3	25,53209432	20,75259209	2,779502233	0,025
	Secretome	3	26,69162051	22,79374758	3,897872925	

Table 4. The RT-PCR result of Osteocalcin.

*P<0,05 means there is significant difference.

On both control and secretome groups, the highest osteocalcin expression were found on the 14th day of observation. The expression then decreased on 21st day. Statistical analysis using

the one-way ANOVA showed a significant difference in both groups (P<0,05). Post hoc test showed that there was a significant difference between the 7th, 14th and 21st days observation in both groups. (see table 5).

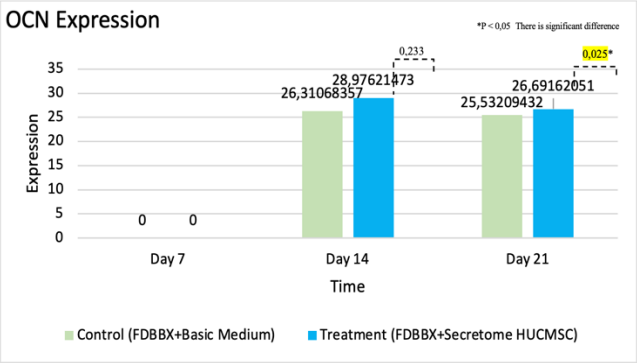

Figure 4. RT-PCR results for osteocalcin expression. The average expression of osteocalcin in secretome group was higher than in control group on the 14th and 21st days. On the 7th day, both control and secretome groups show no osteocalcin expression. Statistical analysis using Independent T test showed that there is significant difference between two group on days 21.

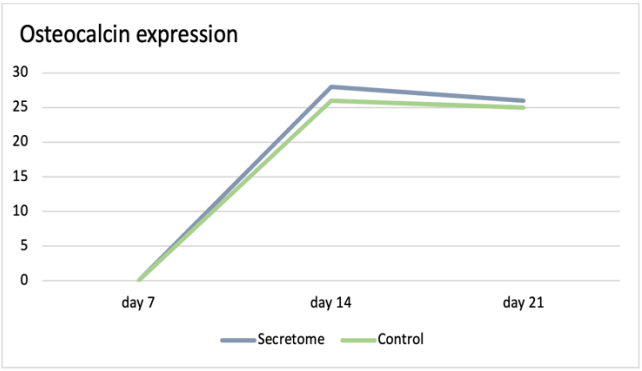

Figure 5. Activity of osteocalcin expression from day 7-21. The highest osteocalcin expression on both control and secretome group were found on 14th day of observation. The expression then decreased on the 21st day.

Day/Day		Osteogenic Medium (Control)			*Secretome* HUCMSC		
		Sig. (Anova)	Sig. (LSD)	Std. Error	Sig. (Anova)	Sig. (LSD)	Std. Error
7	14	0,000	0,000	0,47729	0,000	0,000	1,49739
	21		0,000	0,47729		0,000	1,49739
14	7						
	21		0,152	0,47729		0,178	1,49739
21	7						
	14						

Table 5. Osteocalcin Expression Statistical Analysis.

Discussion

This study used the combination of secretome HUCMSC and three dimensional FDBBX scaffold as a medium for MC3T3-E1 preosteoblast cells to attach and grow. The HUCMSC secretome is expected to enhance the osteogenesis ability of the FDBBX scaffold through the expression of Runt Related Transcription Factor 2 (RUNX2) and Osteocalcin (OCN).

The principle of bone tissue engineering is to create ideal new bone growth with a combination of cells, growth factors and scaffolds that fit the osteoinductive and osteoconductive criterias.[13] Several studies have proven that culture conditions and cell density affect the activity and osteogenic ability of cells.[14] The profile of preosteoblast cell is influenced by several factors, including cell origin (bone marrow, adipose or umbilical), culture condition (2-dimensional, 3-dimensional), and other mechanical stimulus.[12] Research by Yazid et al. (2019) and Li et al. (2017) showed that the MC3T3-E1 cell that was seeded into three dimensional scaffold is more sustainable and exhibit better maturation and proliferative abilities. The study also explained that a higher seeding density was not necessarily better in ostegenic capacity.[14,15] The cell density in this study was 200.000 cells and seeded on a 5x5x3 mm scaffold.

In this study, the RUNX2 expression in the secretome group was higher than the control group on the 14th and 21st days of observation (P<0,05) (figure 2). This result is consistent with the tissue engineering principle. The FDBBX is known for its osteoinductive and osteodifferentiation potential because it has organic components, such as collagen, non-collagen fibers, and growth factors, such as transforming growth factor β (TGFβ) and BMP-2.[7] Human mesenchymal stem cell secretome also contains growth factors (such as IGF, EGF, FGF, and TGF-β), cytokines, and RNA that increase the osteogenic capacity of MC3T3-E1 preosteoblast cells.[12,16] Secretome could promote the osteogenic ability of MSCs, and the effects of secretome often increases with increasing concentrations.[17] FGF-2 and BMP-2 enhance osteogenic differentiation through upregulated the gene expression of the early osteogenic markers, including BMP-2, osterix, and Runx2,

and the late osteogenic markers, osteocalcin and osteopontin.[18]

The highest activity of RUNX2 expression occurred at the beginning of the observation. The highest RUNX2 expression in control group occurred on the 7th day and gradually decreased after 14th and 21st day (figure 3). The osteogenic capacity in this group is dominated by the role of TGF-β and BMP-2.[7] These results are similar to a study by Min et al. (2020) which showed that BMP-2 triggers the osteogenic differentiation and increases RUNX2 expression on day 7.[19] RUNX2 plays crucial role in the proliferation and differentiation of osteoblasts and best known as the master regulator.[20,21] RUNX2 alongside with Sp7 and Wnt induce the differentiation of preosteoblasts to immature osteoblasts. In immature osteoblasts, RUNX2 induce the expression of bone matrix protein genes, including Col1a1, Col1a2, Spp1 and the osteoblasts maturate and produce abundant bone matrix protein.[22]

The result relating to osteocalcin expression showed significant difference between control and secretome group on day 21 (see figure 4). The increase expression was strongly related to secretome application to scaffold FDBBX. This finding is consistent with a study by Gromolak et al. (2020) who stated that the application of BMP-2 and FGF to mesenchymal stem cells increased the osteocalcin expression.[18] Another study by Zhang et al. (2022) also showed that exposure of secretome mesenchymal stem cells increased the osteocalcin expression on polylactic glycolic acid nano particles.[23] Various growth factors from the HUCMSC secretome have several roles in differentiation and maturation of osteoblasts. TGF-β increases the osteogenic markers by binding to type 1 and type 2 receptor which causes the activation of common Smad (Smad 1,5,8). Common Smad alongside with Smad4 interacts with P300 transcription factor dan Crebs Binding Protein resulting in specific gene expression. TGF-β signaling pathways also promote gene expression through the activity of P38 mitogen activated protein kinase (P38MAPK) and phosphoinositide 3 kinase (PI3K).[24] The FGF/FGF receptor (FGFR)-mediated signalling cascade regulates osteoblasts progenitor proliferation, maturation, and apoptosis. FGF/FGFR signalling is reported to induce RUNX2 activation, leading to enhanced expression of osteoblastogenic markers, such as ALP, OCN, and COL1A1, through downstream signaling of phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT), phospholipase γ/PKCα and extracellular receptor kinase (ERK)1/2.[25]

On the 7th day of observation, both study groups show no expression of osteocalcin. Osteocalcin production in the osteoblast lineage is high at later stages of differentiation, and is generally regarded as a marker of “late” osteoblasts.[8] Osteocalcin (OCN) is specifically expressed in mature osteoblasts and is the most abundant non-collagenous protein in bone. Ocn acquires a high affinity to Ca^{2+}.[26] The study from Tsao et al. (2017) confirmed the influence of osteocalcin on hydroxyapatite formation by demonstrating that osteocalcin modulates the mineral species maturation during osteogenic differentiation. Moreover, osteocalcin not only is one of the late protein products of osteogenesis but also a signal to modulate the expression of transcription factors for osteogenic differentiation.[27]

In the 14th and 21st day groups, both RUNX2 and osteocalcin had similar expression patterns. An increase in RUNX2 expression is also followed by an increase in osteocalcin expression. The relationship between RUNX2 and OCN ws explained by Yang et al. (2019) which stated that RUNX2 directly repress miR-31 expression, which significantly inhibits expression of the osteogenic transcription factors OPN, BSP, OSX, and OCN.[28] Komori (2020) also stated that knockdown of RUNX2 gene (RUNX2−/−) on mice show no osteocalcin expression, otherwise the overexpression of RUNX2 induced OCN expression in C3H10T1/2 multipotent mesenchymal cells.[26]

Conclusions

Within the limitation ot this study, it is concluded that the application of HUCMSC secretome could increase the osteoinduction potential of FDBBX scaffold by increasing RUNX2 and osteocalcin expression. Further research is needed to evaluate the osteoinductive potential of the FDBBX scaffold with the HUCMSC secretome in vivo.

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Conflict of Interest

The authors declare that there is no conflict of interest in this study.

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 (024/HRECC.FODM/1/2022), 2022.

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