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Chondrogenic Differentiation Capacity of Human Umbilical Cord Mesenchymal Stem Cells with Platelet Rich Fibrin Scaffold in Cartilage Regeneration (*In Vitro* Study)

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Background: Human umbilical cord mesenchymal stem cell is a promising source of allogenous MSC with great chondrogenic differentiation capacity. Meanwhile, platelet rich fibrin (PRF) is a natural fibrin matrix, rich in growth factors, forming a smooth and flexible fibrin network, supporting cytokines and cell migration, thus can be used as a scaffold that facilitate the differentiation of MSC. However, the differential capability of MSC cultured in PRF was still poorly understood. **Method:** We studied in vitro differentiation potential of MSC cultured in PRF by evaluating several markers such as FGF 18, Sox 9, type II collagen, aggrecan in 3 different culture medium. **Result:** The result showed that there was positive expression of FGF 18, Sox 9, type II collagen, aggrecan in all medium of in vitro culture. **Conclusion:** MSC cultured from human umbilical cord had the capacity of chondrogenic differentiation and able to produce cartilage extracellular matrix *in vitro* which means that hUCMSC is a potential allogeneic MSC for cartilage regeneration.

Keywords: umbilical cord, mesenchymal stem cell, chondrogenic medium.

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INTRODUCTION

Mandibular condylar cartilage is a fibrocartilage tissue which like all type of cartilage, has a low regenerative capacity. This was due to avascular, alymphatic, aneural nature and chondrocyte as the cellular component of articular cartilage was is low turnover in maintaining extracellular matrix.[1,2] Chondrocyte was isolated one to another by dense extracellular matrix that nutrition transport and metabolic waste removal occur through diffusion between extracellular matrix and synovial fluid, caused limited healing capacity after cartilage destruction that need to be reconstructed.[3,4]

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The primary goals of cartilage reconstruction are to relief pain, restoring joint function and to prevent or limiting further joint damage.[5] There are various reconstruction techniques applied in orthopedics and oral and oral maxillofacial surgery such as subchondral drilling, abrasion, micro fracture, mosaic plasty with osteochondral graft, and autologous chondrocyte implantation (ACI) or matrix assisted autologous chondrocyte implantation (MACI) with main purpose to obtain progenitor cells to regenerate cartilage defect. Those techniques were not yet proved to be successful in term of long term functional cartilage regeneration.[6]

Recent approach in cartilage regeneration is using mesenchymal stem cell (MSC) as cartilage progenitor. However, there are several limitations of autologous MSC such as limited cell's availability, donor site related morbidity, repeated surgery, and age dependent proliferative potential. All these factors underlie the choice of allogenous MSC as alternative approach. Adult MSC was proved to be immunoprivilege, immunotolerant, and capable of supressing local immune reaction.[7] Human

umbilical cord mesenchymal stem cells is a promising source of allogenous MSC because it is procured easily and obtained from biological waste thus did not lead to ethical issues.[8] Human umbilical cord mesenchymal stem cells also capable to differentiate into fibrocartilage lineage, forming type I and type II collagen, and aggrecan synthesis.

However, tissue regeneration requires sufficient cell number and scaffold as a support to cells and growth factors.[9] Platelet rich fibrin (PRF) is a natural biomaterial that can produce easily from autologous blood that was centrifuged without anticoagulant, forming fibrin matrix which polymerized naturally and slowly. PRF is rich in growth factors such as PDGF-β, TGFβ-1, VEGF, IGF-I, leukocyte; cytokine such as IL-1β, IL-6, IL-4, and TNFα; and circulating stem cell.[10,11,12] Fibrin matrix contained in the PRF is flexible, elastic and very strong. Platelet rich fibrin consist of weak thrombin concentration with equilateral bond forming a fibrin network that is smooth and flexible, supporting cytokines and cell migration and can be used as a scaffold that facilitate the differentiation of MSC.[13]

Therefore, this study aims to evaluate the capability of in vitro PRF to support chondrogenic differentiation of human cord blood mesenchymal stem cell (hUCMSC).

MATERIAL AND METHODS

Procurement of Human Umbilical Cord

Umbilical cord was procured from cesarean section delivery on a healthy full term pregnancy in the Central Operating Theatre of Dr. Soetomo General Hospital in Surabaya Indonesia. This procedure was legally and ethically approved by Committee for Ethics on Health Research of Dr. Soetomo General Hospital. After the baby had delivered, 2 cm of umbilical cord was cut, washed in Phosphate Buffer Saline (PBS) three times to remove blood excess and blood clot, then soaked in Ringer Lactate solution containing 2.5 µg/mL gentamycin and 1000 U/mL amphotericin B for 20 minutes. This technique was in accordance to Hendrijantini *et al* (2015) procurement technique.[14]

Isolation and Culture of hUCMSC

Isolation and culture procedure were performed at Stem Cell Research and Development Center, Airlangga University, Surabaya. Umbilical cord was minced into small pieces roughly 0.5 cm in size after removal of umbilical arteries and vein and used to isolate the primary culture of hUCMSCs.

Minced cord was enzymatically digested through trypsinization with 0.25% trypsin in 37˚C for 40 minutes then centrifuged, after supernatant removal this process was then repeated twice. The crushed and digested sample was put into PBS containing 0.75 mg/ml collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) and 0.075 mg/mL DNase I (Takara Bio, Shiga, Japan), incubated at 37˚C for 60 minutes. This was followed by filtration with cell strainer and pellet collection upon centrifugation for 10 minutes to finally obtain cells. Singlet cells were collected and cultured with1 x 10^6 cells/cm^2density on collagen-coated dishes using 0,177 *ficol histopaque* (Sigma-Aldrich, St. Louis, MO, USA), *α-modified essential medium* (α-MEM), 20%*fetal bovine serum* (FBS) (Biowest), 100 U penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and amphotericin (Sigma-Aldrich, St. Louis, MO, USA). The medium was changed every three days and when the confluence reached 80% the cell splitting was done using trypsin. Attached cells with 1 x 10^4 cells/cm^2density were replated with the same medium onto new dish to be expanded .[15]

Phenotypic Characterization of hUCMSC

A. Immunocytochemistry

Cultured cells were plated onto coverslips, and after incubated at 37˚C temperature for 1 - 2 hours and fixed with 10% formaldehyde for 15 minutes. Then the coverslips were rinsed four times with PBS and let dried for few minutes. Cells were blocked with PBS and 1%FBS for 15 - 30 minutes and washed with PBS four times. FITC-labelled monoclonal antibody anti-human CD105 and CD45 was applied to the cells and incubated for 60 minutes. Thereafter, the cells were rinsed with PBS twice and they were ready for analysis using fluorescence microscope.

B. Flowcytometry

Cultured hUCMSCs were trypsinized and suspended in *α*-MEM, washed with PBS, fixated in 10%formaldehyde for 10 minutes and stopped with 10% BSA for one hour. Cells were incubated using Human MSCs Analysis Kit (BD Stemflow TM, BD Biosciences) consist of primary antibodies mouse anti-human CD73, CD90, CD105, and negative cocktail containing CD45, CD34, CD19, CD11b, and HLA-DR for 40 minutes. Unbound antibodies were removed by washing with PBS. The bounded primary antibodies were labelled using FITC-conjugated anti-mouse antibody and incubated for 30 minutes. Cells visualized and analyzed using FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Platelet Rich Fibrin Preparation

Blood were aspirated from rats' tail after the rat was anesthetized with 20 mg/body weight ketamine and 3 mg/body weight xylazine injection. One point five ml blood were aspirated with 3 ml disposable syringe, placed in test tube without anticoagulant and centrifuged at 3000 rpm for 10 minutes until formation of 3 separate layers. After removal of top yellowish layer and blood clot from the bottom layer, middle PRF layer was isolated.

In vitro Chondrogenic Differentiation Analysis of hUCMSC with PRF Scaffold

The analysis was performed in 3 groups, consist of the following: 2 experiment groups and 1 control group. In the experiment group hUCMSC and PRF scaffold were placed in culture plate containing ITS plus, 2 mM L-glutamin, 100 µg/ml pyruvate sodium, 0.2 mM ascorbic acid- 2 phosphate, 10^{-7} M dexamethasone (GeneTex, USA), 10 ng/ml TGF-β3, and *high glucose-Dulbecco's Modified Eagle Medium* (DMEM-HG) in first experiment group and *low glucose-Dulbecco's Modified Eagle Medium* (DMEM-LG) in second experiment group. Medium in control group was made of the same component and placed in culture plate containing expansion medium (αMEM). Medium in all groups were changed every 3 days [16]. Chondrogenic differentiation was evaluated after 28 days of culture by immunohistochemistry staining with FGF 18, Sox 9, type 2 collagen, and aggrecan.

RESULTS

Isolation and Culture of hUCMSCs

Cell growth were observed after 24 hours, cells appeared in spindle shaped showing fibroblast like appearance. Cell colony was formed at first passage after 80% confluence was reached. At the fourth passage, monolayer cell formed swirling pattern (**Figure 1**).

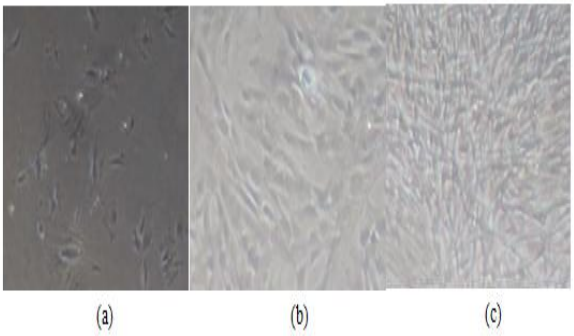

Figure 1.
Culture of hUCMSCs. (a). Cell growth after 24 hours. Cells appeared in spindle shaped showing fibroblast like appearance (inverted microscope, 100 x magnification), (b). cell colony was formed at first passage after 80% confluence (inverted microscope, 100 x magnification), (c). in fourth passage monolayer cell formed swirling pattern (inverted microscope, 40 x magnification).

Phenotypic Characterization of hUCMSCs

A. Immunocytochemistry

Immunocytochemistry examination showed negative expression of CD45 that was hematopoietic stem cell marker, and strong positive expression of CD105 that was mesenchymal stem cell marker (**Figure 2**).

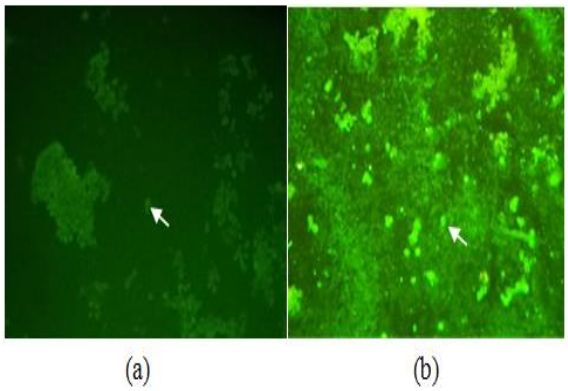

Figure 2.
Phenotypic characterization of hUCMSCs.The immunocytochemistry staining result showed: (a) cells negatively expressed CD45 (white arrow), and (b) cells strongly expressed CD105 (white arrow) under fluorescence microscope, 100 x magnification.

B. Flowcytometry

Flowcytometry analysis was performed after fourth passage. The result showed expression of $CD90^+$ neg PE was 29.80% of subpopulation analyzed, CD 105^+ neg PE was 40.12% of subpopulation analyzed, and $CD73^+CD015^-$ was 32.20% of subpopulation analyzed. Negative results were observed from negative PE markers examination using MSC cocktail containing CD45, CD34, CD11b, CD19, and HLA-DR, as shown in **Figure 3**.

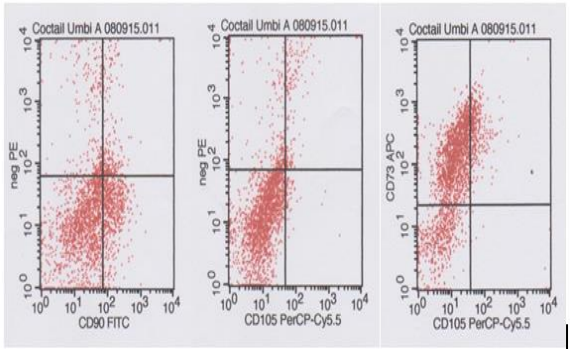

Figure 3.
Flowcytometry analysis results. The results showed expression of $CD90^+$, CD 105^+, $CD73^+$, and negative PE markers.

In vitro chondrogenic differentiation analysis of hUCMSC with PRF scaffold

After 28 days' culture, hUCMSC showed positive expression of FGF 18, Sox 9, type II collagen, and aggrecan that were used as chondrogenic differentiation markers in all groups. The percentage of positive marker expression is presented in **Figure 4.**

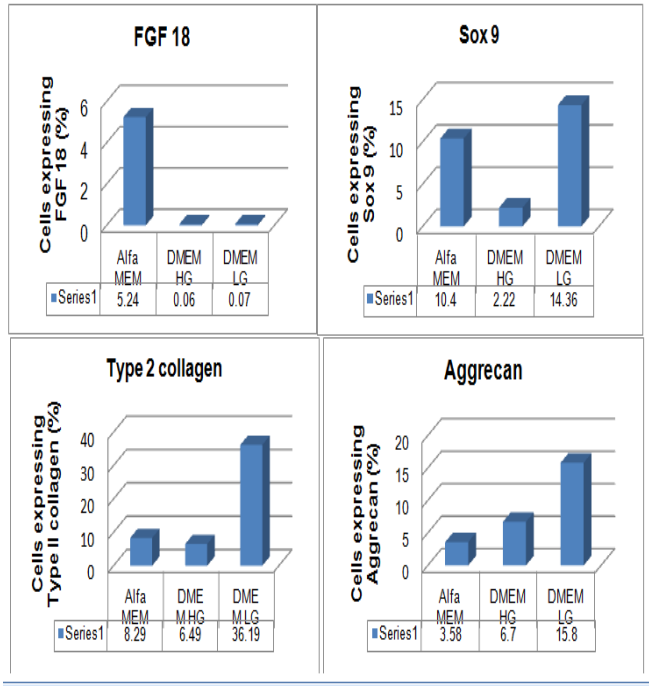

Figure 4.
Percentages of positive expression of FGF 18, Sox 9, type II collagen, and aggrecan in α-MEM, DMEM-HG, and DMEM-LG medium.

Cells expressing FGF 18, Sox 9, type II collagen, and aggrecan in immunohistochemistry staining of hUCMSC and PRF scaffold cultured in αMEM medium is presented in **Figure 5.**

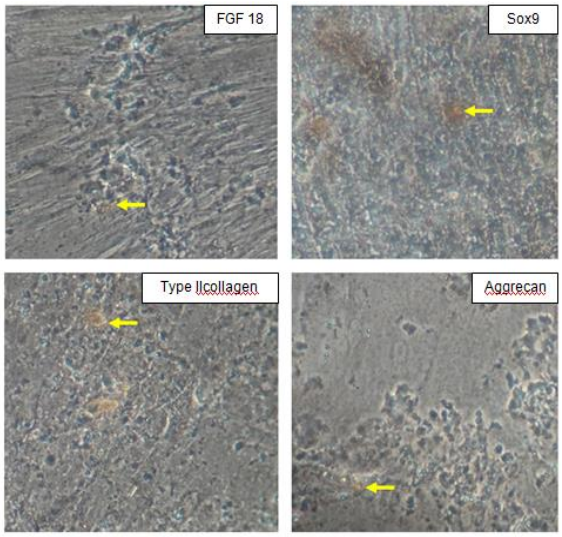

Figure 5.
Immunohistochemistry staining of FGF 18, Sox 9, type II collagen, and aggrecan in α-MEM medium. Arrows represent cells with positive marker.

Positive cells expressing FGF 18, Sox 9, type II collagen, and aggrecan in immunohistochemistry staining of hUCMSC and PRF scaffold cultured in DMEM-HG medium is presented in **Figure 6**.

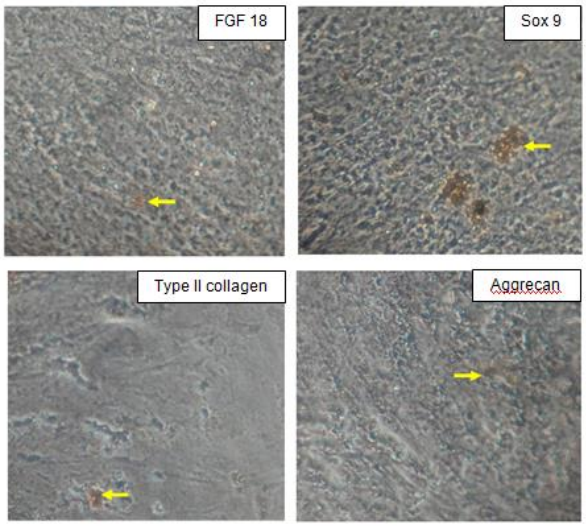

Figure 6.
Immunohistochemistry staining of FGF 18, Sox 9, type II collagen, and aggrecan in DMEM-HG medium. Arrows represent cells with positive marker.

Positive cells expressing FGF 18, Sox 9, type II collagen, and aggrecan in immunohistochemistry staining of hUCMSC and PRF cultured in DMEM-LG medium is presented in **Figure 7.**

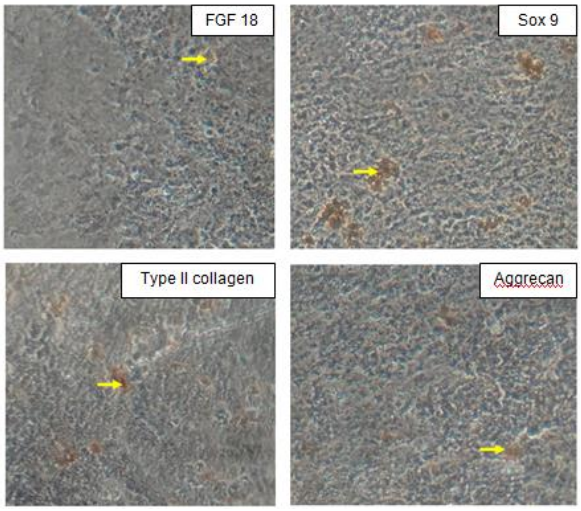

Figure 7.
Immunohistochemistry staining of FGF 18, Sox 9, type II collagen, and aggrecan in DMEM-LG medium. Arrows represent cells with positive marker.

DISCUSSION

Human umbilical cord was procured from cesarean section delivery under highly aseptic condition to avoid microorganism contamination, especially fungi. Kamadjaja *et al* (2014) strongly advised not to procure tissue sample from normal delivery because of high possibility of fungal overgrowing in cell culture.[17]

Isolation and culture protocol was performed in accordance to protocol of Stem Cell Laboratory, Institute of Tropical Disease, Airlangga University. Enzymatic digestion was used in MSC isolation because this method was efficient in isolation and culture of MSC.[18] Primary cells from this isolation method showed MSC character, which was spindle shaped resembling fibroblast, plastic adherence, and formed swirling pattern when confluence was reached (**Figure 1c**).

Phenotypic characterization of hUCMSC with immunocytochemistry staining using CD105 and CD45 showed strong expression of MSC marker CD105 and negative expression of hematopoietic stem cell marker CD45. Flowcytometry analysis showed the expression of $CD90^+$, CD105, $CD73^+$, $CD34^-$, and $CD45^-$. Those findings met the minimal criteria of MSC determined by International Society for Cellular Therapy (ISCT).[19] In addition, characterization analysis was performed at late passage still showed MSC character. Zhuang *et al* (2015) reported that there was no phenotype expression difference in early or late passage of hUCMSC, referring that there was no change in MSC character.[20]

Chondrogenic differentiation was observed after 28 days of *in vitro* culture in all medium used in this study. Interesting finding was FGF-18 expression was high in αMEM culture medium group might be caused by slower differentiation of hUCMSC into chondrocyte compared with FGF-18 expression in DMEM-HG and DMEM-LG. FGF-18 increased Sox 9 expression and therefore increase MSC differentiation and work early in the chondrogenesis. We suggest that low FGF-18 expression in both chondrogenic medium might be caused by hUCSMC that already differentiated into chondrocyte and formed cartilage matrix.[21] Those finding was in accordance to literature report by Liu (2007) that presented evidence of FGF-18 signaling importance in early chondrocyte proliferation.[22]

Positive expression of Sox-9 after 28 days' culture establishing the role of Sox-9 in maintaining chondrocyte phenotype which underlie the high expression of Sox-9 in fully differentiated chondrocyte.[23] Highest expression of Sox-9, type 2 collagen, and aggrecan was observed in the DMEM-LG medium compared to those cultured in αMEM an DMEM-HG culture medium. Those findings showed that there were higher chondrogenic differentiation of hUCMSC in DMEM-LG medium and was consistent with other study performed by Tsai (2013). High glucose culture medium reduced hMSC responsiveness to chondrogenic induction that result in lower TGFβRII and thereby decreased the activation of downstream signaling molecules during chondrogenesis.[24] Heywood *et al* (2014) reported that the expression of chondrocyte differentiation markers was preserved better and irreversible in low glucose condition. Thus, increase subsequent cartilage formation in 3D pellet culture. Chondrocytes initially proliferated faster under high glucose condition but stopped earlier compared to chondrocyte in low glucose condition. This phenomenon possibly caused by cumulative cell stress experienced by rapidly proliferating MSC. Higher longevity of chondrocyte *in vitro*indicated that the expansion of MSC in such medium was also considerably high under low glucose condition.[25]

CONCLUSION

The conclusion based on this study was MSC cultured from human umbilical cord had the capacity of chondrogenic differentiation and able to produce cartilage extracellular matrix *in vitro* which means that hUCMSC is a potential allogeneic MSC for cartilage regeneration.

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REFERENCES

1. Tanaka E, Detamore MS, Mercuri LG, 2008. Degenerative Disorders of the Temporomandibular Joint: Etiology, Diagnosis, and Treatment. *J Dent Res* 2008;87(4): 296–307.
2. Wang L, Lazebnik M, Detamore MS. Hyaline cartilage cells outperform mandibular condylar cartilage cells in a TMJ fibrocartilage tissue engineering application. *Osteoarthitis Cart* 2009;17(3): 346-53.
3. Goldring MB, Otero M, Plumb DA, Dragomir C, Favero M, El Hacem K, Hashimoto K, Roach HI, Olivotto E, Borzi RM, Marcu KB. Roles of inflamatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. *European Cell Mat* 2011;21: 202-20. NIHMSID: NIHMS349842
4. Zhang L, Hu J, Athanasiou KA, 2009. The role of tissue engineering in articular repair and regeneration. *Crit Rev Biomed Eng* 2009;37(1-2): 1-57.
5. Naujoks C, Meyer U, Weismann HP, Jäsche-Meyer J, Hohoff A, Depprich R, Handschel J. Principles of cartilage tissue engineering in TMJ reconstruction. Head Face Med 2008;4(3): 1-7.
6. Punwar S, Khan WS. Mesenchymal stem cells and articular cartilage repair: clinical studies and future direction. The Open Orthop J 2011;5(suppl 2 M-11): 296-301.
7. Buja LM, Vela D. Immunologic and inflammatory reactions to exogenous stem cells. Implications for experimental studies and clinical trial for myocardial repair. J Am Coll Cardiol 2010;56: 1693-700.

8. Lindenmair A, Hatlapatka T, Kollwig G, Hennerbichler S, Gabriel C, Wolbank S, Redl H, Kasper C. Mesenchymal stem or stromal cells from amnion and umbilical cord tissue and their potential for clinical applications. Cells 2012;1:1061-88.
9. Horst OV, Chavez MG, Jheon AH, Desai T, Klein OD. Stem cell and biomaterials research in dental tissue engineering and regeneration. *Dent Clin North Am* 2012;56: 495-520.
10. Kazemi D, Fakhrjou A, Dizaji VM, Alishahi MK. Effect of autologus platelet rich fibrin on the healing of experimental articular cartilage defects of the knee in an animal model. *Biomed Res Int*. 2014: 1-10..
11. Baek HS, Lee HS, Kim BJ, Chung IK, Kim CH, Jin SM, Hwang HS, Shin SH, 2011. Effect of platelet-rich fibrin on repair of defect in the articular disc in rabbit temporomandibular joint by platelet-rich fibrin. *Tissue Eng Reg Med* 2011;8(6):530-5.
12. Saluja H, Dehane V, Mahindra U, 2011. Platelet-rich fibrin: a second generation platelet concentrate and a new friend of oral and maxillofacial surgery. *Ann Maxillofac Surg* 2011;1(1): 53-7..
13. Khiste SV, Tari RN. Platelet-rich fibrin as a biofuel for tissue regeneration. *ISRN Biomaterials* 2013: 1-6.
14. Hendrijantini N, Kresnoadi U, Salim S, Agustono B, Retnowati E, Syahrial I, Mulawardhana P, Wardhana MP, Pramono C, Rantam FA. Study biocompatiblity and ostegenic differentiation potential of human umbilical cord mesenchymal stem cells (hUCMSCs) with gelatin solvent. *J Biomed Sci Eng* 2015;8: 420-8.
15. Rantam FA, Ferdiansyah, Purwati, 2014. Stem cell mesenchymal, hematopoetik dan model aplikasi. 2nd Ed. Surabaya: Airlangga University Press, P. 38.
16. Sudo K, Kanno M, Miharada K, Ogawa S, Hiroyama T, Saijo K, Nakamura Y, 2007. Mesenchymal progenitor able to differentiate into osteogenic, chondrogenic, and/or adipogenic cells in vitro are present in most primary fibroblast-like cell populations. *Stem* Cells 2007;25: 1610-7.
17. Kamadjaja DB, Purwati, Rantam FA, Ferdiansyah & Pramono C. The osteogenic capacity of human membrane mesenchymal stem cell (hAMSC) and potential for application in maxillofacial bone reconstruction in vitro study. *J Biomed Sci Eng* 2014;7: 497-503.
18. Han YF, Tao R, Sun TJ, Chai JK, Xu G & Liu J,. Optimization of human umbilical cord mesenchymal stem cell isolation and culture methods. *Cytotechnology* 2013;65: 819-27.
19. Dominici M, Le Blanc K Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ & Horwitz EM,. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cryotherapy* 2006;8(4): 315-7.
20. Zhuang Y, Li D, Fu J, Shi, Lu Y & Ju X, 2015. Comparison of biological properties of umbilical cord-derived mesenchymal stem cells from early and late passages: immunomodulatory ability is enhanced in aged cells. *Mol Med Rep* 2015;11: 166-74.
21. Elluru R, Thompson F, & Reece A,. Fibroblast growth factor 18 gives growth and directional cues to airway cartilage. *Laryngoscope* 2009;119(6):1153-65
22. Liu Z, Lavine KJ, Hung IH, & Ornitz DM,. FGF 18 is required for early chondrocyte proliferation, hypertrophy and vascular invasion of the growth plate. *Dev Biol* 2007;302: 80-91
23. Lefebvre V and Crombrugghe BD,. Toward understanding Sox 9 function in chondrocyte differentiation**.** *Matrix Biology* 1998;16: 529-40. PMID: 9569122.
24. Tsai TL, Manner PA, & Li WJ,. Regulation of mesenchymal stem cell chondrogenesis by glucose through protein kinase C/transforming growth factor signaling. *Osteoarthritis Cartilage* 2013;21: 368-76
25. Heywood HK, Nalesso G, Lee DA, & Dell'Accio F,. Culture expansion in low-glucose conditions preserves chondroyte differentiation and enhances their subsequent capacity to form cartilage tissue in three-dimensional culture. *BioRes* 2014;3(1): 9-18