International Journal of Medical Laboratory 2020;7(4):280-288.

Original Article

Which Culture System Is better for Chondrogenesis of Adipose-Derived Stem Cells ; Pellet or Micromass?

Mahtab Teimouri[1] Ph.D., Batool Hashemibeni[2*] Ph.D., Mohammad Mardani[2] Ph.D., Muhammadhosein Moradi[3] M.D. Student

[1] Department of Anatomical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2] Department of Anatomical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran
[3] Student Research Committee, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Article history
Received: 21 Apr 2020
Accepted: 23 Aug 2020
Available online: 30 Nov 2020

Keywords
Chondrogenesis
Collagen
Stem cell

A B S T R A C T

Background and Aims: The current study was conducted to compare the expression levels of collagen type Π and X during chondrogenesis of human adipose-derived mesenchymal stem cells (hADMSCs) pellet and micromass cultures.

Materials and Methods: Extracted hADMSCs were cultured until three passages and then transferred to pellet and micromass cultures in the experimental groups of day 7 and day14. For pellet and micromass cultures, aliquots of 5×10^5 cells/ml were centrifuged and respectively cultured in the conical tubes and droplets (12.5 µl) of the 24-well plates containing chondrogenic medium. Realtime-polymerase chain reaction technique was performed for gene expression levels.

Results: Increased expression of collagen type Π was shown in micromass day14 compared to micromass day 7, pellet day 14 ($p<0.01$) and pellet day 7 ($p<0.001$). Also, an increased expression of collagen type Π was seen in micromass day 7 and pellet day 14 compared to pellet day 7 ($p< 0.05$). Expression of collagen type X increased in pellet day 14 compared to micromass on days 7 and 14 ($p<0.001$, $p<0.01$) and pellet day14 compared to pellet day7 ($p< 0.05$). An increased expression of collagen type X was shown in pellet day 7 compared to micromass on days 7 and 14 ($p<0.05$).

Conclusions: According to the results, higher expression of collagen type Π and lower expression of collagen type X in micromass cultures that are prepared by cell suspension play a better role during cellular condensation that leads to the formation of large nodules exhibiting cartilage-like morphology, suggests a higher efficiency for micromass cultures.

[*]**Corresponding Author:** Department of Anatomical Sciences, Isfahan University of Medical Sciences HezarJerib Street, Isfahan, Iran, and **Tel:** +983137929153, **Email:** hashemibeni@med.mui.ac.ir

Introduction

Recently, articular cartilage defects are increasing. These defects lead to the restriction of the musculoskeletal system. Tissue engineering, as a beneficial method, is being used for studying and repairing these defects [1]. There are many effective factors in the successful functional regeneration of the articular cartilage tissues, such as selecting appropriate cell source and cell culture systems [2]. Regarding this purpose, human adipose-derived mesenchymal stem cells (hADMSCs) are one of the appropriate sources for *in vitro* studies. These cells are available in millions of cells per individual and are easily isolated from adipose tissue [3]. A wide self-renewal capability [4] and high potential to be cultured in vitro for several months with a low senility rate can also be mentioned as other advantages [5]. Previous studies showed that monolayer culture could decrease the chondrogenesis potential of mesenchymal stem cells (MSCs) [6]. Transformation of cells into fibroblasts and collagen type I secretion in the extracellular matrix (ECM) instead of expressing the specific markers of cartilage tissue such as collagen type II, and aggrecan can happen [7]. Different three-dimensional (3D) culture systems such as scaffold-based culture systems [8] and scaffold-free culture systems were devised to overcome this issue [9]. Pellet and micromass culture, as the most simple 3D scaffold-free culture systems, are considered successful in the imitation of the embryonic development of cartilage tissue. These culture systems provide a 3D environment similar to embryonic development conditions [10]. Cell masses with high cell density formed after centrifugation and cell suspension in pellet and micromass cultures, similar to the embryonic precartilage condensation, contribute to better chondro-genesis [11]. Also, having extensive cell to cell and cell to ECM connections in pellet and micromass cultures, suggests them appropriate culture systems [12].

Collagen type Π is one of the most important ECM components of the cartilage tissue. Proteoglycan aggregations are trapped in the ECM of the cartilage tissue by this component as a fibrillary network. Collagen type X is normally expressed during endochondral ossification. This collagen expression as a hypertrophic marker of chondrocytes is undesirable in hyaline cartilage tissue during chondrogenesis [13]. The current study was conducted to compare expression levels of collagen type Π and X after chondrogenesis of hADMSCs in pellet and micromass culture systems.

Materials and Methods

Cell isolation

hADMSCs were extracted from three patients following abdominal surgeries (liposuction) after receiving informed consent (Mean age of 35years, AL-Zahra hospital, Isfahan, Iran). Subcutaneous fat tissue samples were digested after mechanical cut-down and washing with phosphate buffered saline (Sigma) with 0.1% collagenase A (Sigma) solution and incubated under 5% CO_2 and 95% humidity at 37˚C for 40 min. To deactivate the collagenase enzyme

activity, the mixture was incubated in an expansion medium containing Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Sigma)+10% fetal bovine serum (FBS) solution (Invitrogen) and 1% penicillin/streptomycin (Gibco). The cell solution was then centrifuged at 1400 rpm for 10 min. The supernatant of the solution containing fat tissue and the medium was removed. Then, cells were suspended in a fresh expansion medium. Afterward, cells were cultured in the tissue culture flask T-75 and placed in the CO_2 incubator with 95% humidity and 5% CO_2 at 37˚C. The culture medium was exchanged every 3-4 days. Third passage cells were counted and assigned into four groups, including the pellet cell culture on days 7 and 14 and the micromass cell culture on days 7 and 14 [14]. The cells were then transferred to scaffold-free three-dimensional culture systems of the pellet and micromass for chondrogenic induction. Chondrogenic culture medium consisted of DMEM high glucose, penicillin & streptomycin 1%, Dexamethasone 10^{-7} M, insulin, transferrin, selenium (ITS) 1%, bovine serum albumin (BSA) 1%, Ascorbate 2 phosphate 50 µg/ml, Linoleic acid 5µg/ml and transforming growth factor (TGF)-β3 10 ng /ml. All chemicals for chondrogenesis culture medium were provided by Sigma-Aldrich (USA), [15].

Cell culture

Pellet culture procedure

The three times passaged hADMSCs were harvested and were suspended at the aliquots of 5×10^5 cells per each pellet culture in conical tubes of 1000 µl chondrogenic medium. Then cell suspensions were centrifuged at 1400 rpm for 10 min in 15-ml polypropylene conical tubes. The centrifuged cell sediment was at the bottom of each tube. Each tube contains cells, and the chondrogenic medium was placed in a humidified incubator with 5% CO_2 at 37˚C. For gases to be exchanged, the caps of pellets were loosened 24 hours after incubation. Sediment cells became a spherical accumulation on the floor of each conical tube. The culture medium was changed every 3-4 days. Pellet cultured cells were harvested on days 7 and 14 [16].

Micromass culture procedure

Cultured cells at passage 3 were isolated at a density of 5×10^5 cells per each well plate and were suspended in 12.5 µl of the chondrogenic medium. Then, cell droplets (12.5 µl) were carefully placed in each well of the 24-well plate. The cultured cells were kept in the incubator for 2 hours to adhere to the plate wells' floor. After that, 0.5 ml chondrogenic medium was added to each well. After 24 hours, the cell droplets shuffled and became round in form. The chondrogenic medium was exchanged every 3-4 days. Micromass cultured cells were harvested on days 7 and 14 [16].

Analysis of genes by real-time polymerase chain reaction (PCR) assay

At first, cultures were washed and placed in a digestion solution (Merck). The resultant solution was centrifuged at 1400 rpm for 5 min. Extracted cells were used for RNA extraction by an RNeasy mini kit (Qiagen). The extent of extracted RNA was measured by spectro-photometer (Bio photometer, Eppendorf) at 260/280 nm wavelength. Total extracted RNA was used to synthesize cDNA by the recruitment of Revert Aid TM First Strand cDNA Synthesis

Kit (Fermentas). Maxima SYBR® Green/Rox qPCR Master Mix 2X (Fermentas) was used to measure relative quantification of the expression of collagen types II and X. Glyceraldehyde 3-phosphate dehydrogenase(GAPDH) was used as an endogenous control gene. The Realtime-PCR reaction was done for 1 min at 95˚C and 40 amplification cycles (15 sec at 95˚C, 15 sec at 60˚C, and 20 sec at 72˚C). Comparative expression levels for each primer were expressed by the $2^{-\Delta\Delta Ct}$ method [17]. Then, the applied primers for each gene were designed by Allele ID software (Primer Biosoft), (Table1). A general illustration of the current study was considered in figure 1. This study was approved by the Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran.

Table 1. Used primers in Realtime –PCR assay

Gene	Primer sequences	(Size, Base pair)
Collagen II -F	5´ - CTGGTGATGATGGTGAAG-3	130
Collagen II -R	5´- CCTGGATAACCTCTGTGA-3	
Collagen x -F	5´- AGAATCCATCTGAGAATATGC -3´	187
Collagen x -R	5´- CCTCTTACTGCTATACCTTTAC-3	
GAPDH -F	5´- AAGCTCATTTCCTGGTAT -3	125
GAPDH -R	5´- CTTCCTCTTGTGCTCTTG -3	

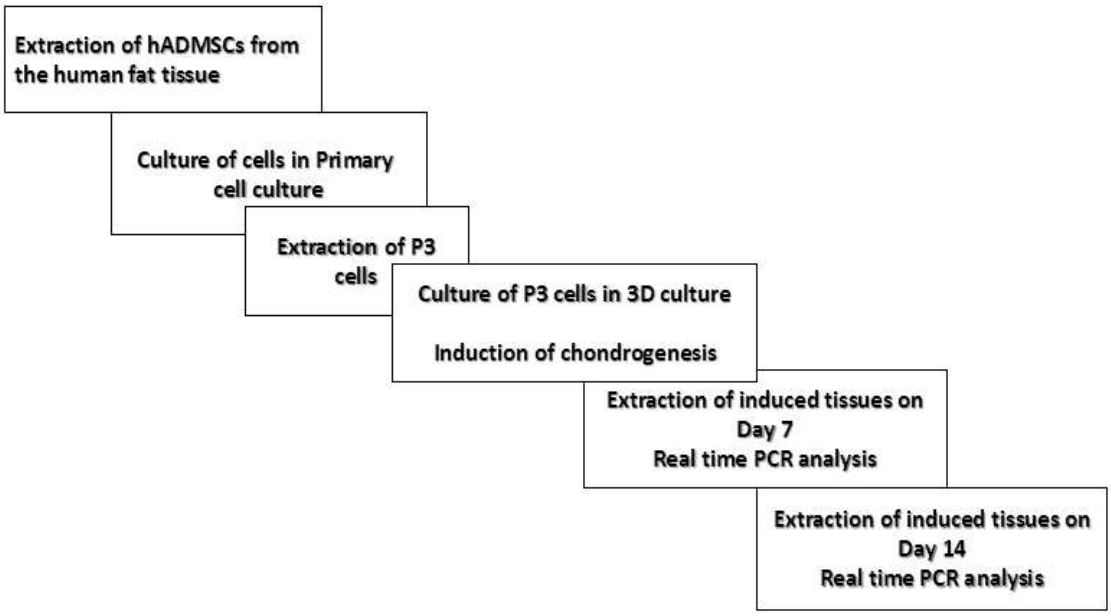

Fig. 1. Graphical abstract of the current study, hADMSCs= Human adipose-derived mesenchymal stem cells; P3= Third passage; 3D= Three dimensional; PCR= Polymerase chain reaction

Statistical analysis

Statistical analysis was carried out by SPSS version 16.0 (copyright© SPSS Inc.2007, US). The Kolmogorov-Smirnov test assessed the normal distribution of data. The one-way analysis of variance (ANOVA) and post-hoc Tukey tests were used to compare realtime-PCR results in the different groups. All data were expressed as means±SEM, and the significant level was set at $p< 0.05$.

Results

Histological features of hADMSCs in monolayer culture

HADMSCs with a fibroblast resembling morphology were adhered to the floor of the culture flask T-75 after several hours (Fig. 2, A). Cells at third passage were confluent and occupied about 80% of the culture flask after 8-12 days (Fig. 2, B).

Histological features of induced-cartilage tissues in the 3D culture system

Over time, due to the synthesis of the ECM and high cell to cell and cell to ECM interactions, induced-cartilage tissue, exhibited a nodule form in the bottom of the conical tube in the pellet culture and a round form at the floor of each well in the micromass culture (Fig. 3, A and B).

Gene expression

Expression of collagen type Π is significantly increased in micromass culture day 14 compared to micromass culture day 7 ($P<0.01$). Also, there is a significantly increased expression of collagen type Π in micromass culture day 14 compared to pellet culture day 14 ($P<0.01$).

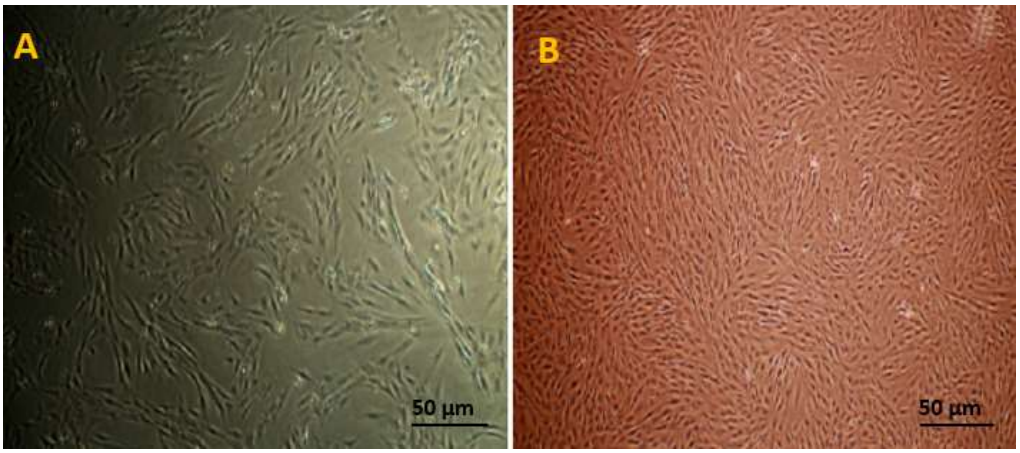

Fig. 2. Inverted microscopic images of cultured hADMSCs in monolayer culture. Cells have a fibroblast-like morphology. A, cells in the first passage. B, cells at third passage that have reached 80% confluency

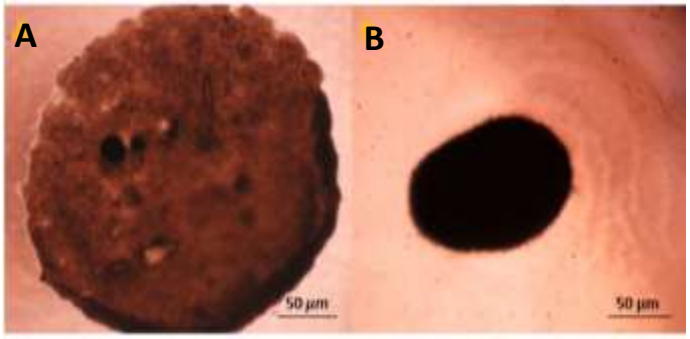

Fig. 3. Inverted microscopic images of the induced-cartilage tissue in micromass and pellet cultures. A, induced- tissue in micromass culture at day14. B, induced-cartilage tissues in pellet culture at day 14

Significantly increased expression of collagen type Π was shown in micromass culture day 14 compared to pellet culture day 7 ($p<0.001$). There is a significant increase in the expression of

collagen type Π in pellet culture day 14 compared to pellet culture day 7 ($p<0.05$). An increased expression of collagen type Π was seen in micromass culture day 7 compared to pellet culture day 7 ($p<0.05$) (Figure 4). As shown in Figure 5, there is a significantly increased expression of collagen type x in pellet culture on day 14 compared to micromass cultures on days 7 and 14 ($p<0.001$, $p<0.01$). Furthermore, there is a significantly increased expression of collagen type x in pellet culture day 14 than pellet culture day7 ($p<0.05$). Our results demonstrated that the expression of collagen type X is significantly increased in pellet culture day 7 compared to micromass cultures on days 7 and 14 ($p<0.05$).

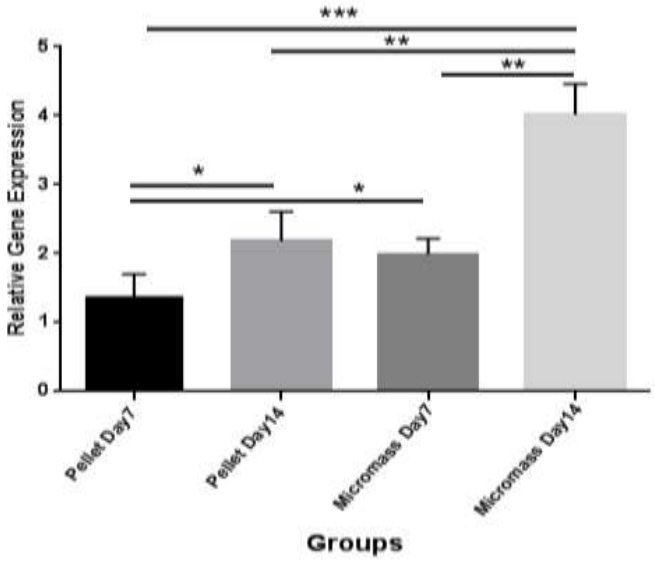

Fig. 4. Real-time -PCR analysis of relative gene expression of collagen type Π of the induced-cartilage tissue in micromass and pellet cultures in the studied groups. There is a significant increase in collagen type Π in micromass cultures compared to the other groups. The bars were presented at means ± SEM, (*** $p < 0.001$, **$p < 0.01$, *$p < 0.05$).

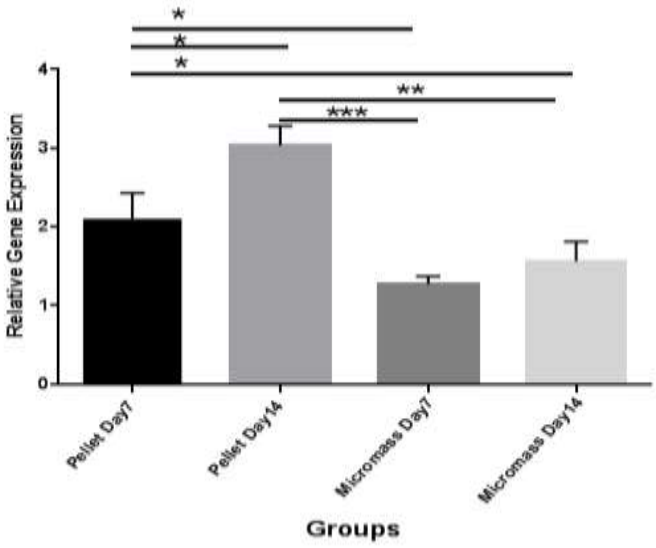

Fig. 5. Real-time -PCR analysis of relative gene expression of collagen type X of the induced-cartilage tissue in micromass and pellet cultures in the studied groups. There is a significant increase in collagen type X in pellet cultures compared to the other groups. The bars were presented at means ± SEM, (*** $p < 0.001$, **$p < 0.01$, *$p < 0.05$).

Discussion

In the current study, the most increased expression of collagen type Π was shown in micromass culture. Also, in our previous study, aggrecan synthesis significantly increased in micromass culture that it can be considered a close correlation of these components of cartilage tissue with each other [16]. Our data showed that higher expression of collagen type X as an undesirable marker of chondrocytes in pellet cultures could cause a low oxygen environment that induces extremely high cell density formed by centrifugation [17]. This condition can lead to the poor cell to cell interactions and little diffusion of nutrients that will finally cause apoptosis and hypertrophy in the central region of pellet cultures [12]. On the contrary, micromass culture, while providing optimal cell density [18], causes an equilibrium between cell density and diffusion of nutrients [19]. Chang et al., in a comparative study between pellet and collagen gel, reported an increase in the expression of the genes involved in chondrogenesis in the collagen gel compared to pellet culture [20]. A previous study has shown an acceptable performance of the pellet culture system as a simple technical method in human intervertebral disc cells' culture. The native phenotype of the human intervertebral disc cells was maintained in this high-density culture system. So, the pellet culture system might be optimal for *in vitro* and *in vivo* biochemical studies for various studies such as tissue engineering and gene therapy [21]. Findings of a comparative study between pellet culture and alginate-bead culture suggested better chondrogenic potential in pellet cultures compared to the alginate-based system [22]. In the current study, decreased expression of collagen type Π in pellet cultures agrees with our previous similar study, showing a reduced expression level of aggrecan in pellet culture [16]. This data can indicate unsuccessful chondrogenesis of hADMSCs in this cell culture system. A previous comparative study between micromass culture and three-dimensional aqueous-derived silk scaffold showed that biocompatible and biodegradable features of three-dimensional aqueous-derived silk scaffold helped improve the chondrogenic differentiation of ADMSCs compared to micromass culture [23]. Another previous study showed that expression level of SOX-9, aggrecan, and type II collagen during chondrogenesis of human amniotic fluid stem cells were significantly increased in micromass culture. It can be considered that micromass culture was presented as an effective culture system, having a high potential for differentiation of human amniotic fluid stem cells into chondrocyte [24]. A previous study showed that micromass culture as a beneficial culture system could successfully differentiate and mineralize murine mesenchymal C3H10T1/2 cells [25]. Another previous study showed that micromass culture was a good tool for tracing and investigating the effects of the essential metals on human chondrocytes *in vitro* [26]. Also, study of Schäfer et al, showed that micromass culture can be effective in investigation of dynamic behavior of osteoblasts during ossification [27]. Basiri et al., showed that micromass culture is an effective culture system in formation of cartilage tissue

from hADMSCs using herbal component [28]. Although, a previous study has compared chondrogenesis of human bone marrow stem cells in pellet and micromass cultures but this study mainly differs from that due to the utilization of hADMSCs instead of human bone marrow stem cells, which are more appropriate cell sources and can be extracted easily with less cost and without invasive methods [29].

Conclusion

According to the findings, higher expression of collagen type Π and lower expression of collagen type X in micromass cultures prepared by cell suspension play a better role during cellular condensation, leading to the formation of large nodules exhibiting cartilage-like morphology suggests a higher efficiency for micromass cultures.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

This study was financially supported by Isfahan University of Medical Sciences (Grant no. 391233). Authors would also like to appreciate the partnership of Dr. Mohsen Mahmoudieh and Dr. Vahid Goharian, fellowships of laparoscopy, and Thorax surgery of Al Zahra and Amin Hospitals in the current research.

References

[1].Go G, Jeong SG, Yoo A, Han J, Kang B, Kim S, et al. Human adipose–derived mesenchymal stem cell-based medical microrobot system for knee cartilage regeneration in vivo. Science Robotics 2020; 5(3): 66-72.

[2]. Setayeshmehr M, Esfandiari E, Hashemibeni B, Tavakoli AH, Rafienia M, Samadikuchaksaraei A, et al. Chondrogenesis of human adipose-derived mesenchymal stromal cells on the [devitalized costal cartilage matrix/poly (vinyl alcohol)/fibrin] hybrid scaffolds. European Polymer Journal 2019; 118(3): 528-41.

[3]. Sharifian Z, Hashemibeni B, Pourentezari M, Valiani A, Mardani M, Rarani MZ, et al. Comparison of PLGA/Fibrin and PLGA/ hyaluronic acid scaffolds for chondrogenesis of human adipose-derived stem cells. Int J Med Lab. 2020; 7(2): 128-37.

[4]. Sanchooli T, Norouzian M, Teimouri M, Ardeshirylajimi A, Piryaei A. Adipose-derived stem cells conditioned media promote in vitro osteogenic differentiation of hypothyroid mesenchymal stem cells. Gene, Cell and Tissue 2020; 7(3): 1-8.

[5]. Amirpour N, Amirizade S, Hashemibeni B, Kazemi M, Hadian M, Salehi H. Differentiation of eye field neuroectoderm from human adipose-derived stem cells by using small-molecules and hADSC-conditioned medium. Annals of Anatomy-Anatomischer Anzeiger 2019; 221(1): 17-26.

[6]. Izadi M, Valiani A, Bahramian H, Esfandiari E, Hashemibeni B. Which factor is better for cartilage tissue engineering from human adipose-derived stem cells? Kartogenin or avocado soybean unsaponifiable. Pharmacophore 2018; 9(1): 140-48.

[7]. Grigull NP, Redeker JI, Schmitt B, Saller MM, Schönitzer V, Mayer-Wagner S. Chondrogenic potential of pellet culture compared to high-density culture on a bacterial cellulose hydrogel. Int J Mol Sci. 2020; 21(8): 2785.

[8]. Hashemibeni B, Pourentezari M, Valiani A, Zamani M, Mardani M. Effect of icariin on the chondrogenesis of human adipose derived stem cells on poly (lactic-co-glycolic) acid/fibrin composite scaffold. Int J Adv Biotech Res. 2017; 8(2): 595-605.

[9]. Valiani A, Hashemibeni B, Esfandiary E, Ansar MM, Kazemi M, Esmaeili N. Study of carbon nano-tubes effects on the chondrogenesis of human adipose derived stem cells in alginate scaffold. Int J Prevent Med. 2014; 5(7): 825-32.

[10]. Zamani S, Hashemibeni B, Esfandiari E, Kabiri A, Rabbani H, Abutorabi R. Assessment of TGF-β3 on production of aggrecan by human articular chondrocytes in pellet culture system. Advanced biomedical research. 2014; 3(1): 54-61.

[11]. Zhao X, Qiu X, Zhang Y, Zhang S, Gu X, Guo H. Three-dimensional aggregates enhance the therapeutic effects of adipose mesenchymal stem cells for ischemia-reperfusion induced kidney injury in rats. Stem Cell Int. 2016; 1(S 1): 1-11.

[12]. Mahboudi H, Kazemi B, Hanaee-Ahvaz H, Ardeshirylajimi A, Eftekhary M, Enderami SE. Comparison between high cell-density culture systems for chondrogenic differentiation and

articular cartilage reconstruction of human mesenchymal stem cells: A literature review. Regener, Reconstruc Restor. 2017; 2(1): 7-15.

[13]. Lehmann R, Gallert C, Roddelkopf T, Junginger S, Jonitz-Heincke A, Wree A, et al. Manually and automatically produced pellet cultures of human primary chondrocytes: A comparative analysis. Engineer Life Sci. 2016; 16(3): 272-82.

[14]. Pelttari K, Winter A, Steck E, Goetzke K, Hennig T, Ochs BG, et al. Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis Rheumatism 2006; 54(10): 3254-266.

[15]. Mardani M, Kabiri A, Esfandiari E, Esmaeili A, Pourazar A, Ansar M, et al. The effect of platelet rich plasma on chondrogenic differentiation of human adipose derived stem cells in transwell culture. Iranian journal of basic medical sciences 2013; 16(11): 1163-169.

[16]. Teimouri M, Hashemibeni B, Mardani M. Comparison of aggrecan gene expression in chondrogenesis of adipose-derived stem cells in pellet and micromass culture systems. Tehran Univ Med J. 2018; 76(2): 90-5.

[17]. Witt A, Salamon A, Boy D, Hansmann D, Büttner A, Wree A, et al. Gene expression analysis of growth factor receptors in human chondrocytes in monolayer and 3D pellet cultures. Int J Molecul Med. 2017; 40(1): 10-20.

[18]. Ghaffari E, Talebpour Amiri F, Karimpour Malekshah A, Mirhosseini M, Barzegarnejad A. Comparing the chondrogenesis potential of human adipose-derived stem cells in monolayer and micromass culture systems. J Mazandaran Univ Med Sci. 2016; 25(133): 37-47.

[19]. Iezaki T, Fukasawa K, Yamada T, Hiraiwa M, Kaneda K, Hinoi E. Cartilage induction from mouse mesenchymal stem cells in high-density micromass culture. Bio-Protocol. 2019; 9(1): 1-6.

[20]. Chang CH, Lin HY, Fang HW, Loo ST, Hung SC, Ho YC, et al. Chondrogenesis from immortalized human mesenchymal stem cells: comparison between collagen gel and pellet culture methods. Artifici Organ. 2008; 32(7): 561-66.

[21]. Liang C, Li H, Tao Y, Zhou X, Li F, Chen G, et al. Responses of human adipose-derived mesenchymal stem cells to chemical microenvironment of the intervertebral disc. J Translat Med. 2012; 10(1): 49-54.

[22]. Ma K, Titan AL, Stafford M, hua Zheng C, Levenston ME. Variations in chondrogenesis of human bone marrow-derived mesenchymal stem cells in fibrin/alginate blended hydrogels. Acta Biomaterialia 2012; 8(10): 3754-764.

[23]. Kim HJ, Park SH, Durham J, Gimble JM, Kaplan DL, Dragoo JL. In vitro chondrogenic differentiation of human adipose-derived stem cells with silk scaffolds. Journal of tissue engineering. 2012; 3(1): 204-207.

[24]. Zuliani CC, Bombini MF, Andrade KC, Mamoni R, Pereira AH, Coimbra IB. Micromass cultures are effective for differentiation of human amniotic fluid stem cells into chondrocytes. Clinics 2018; 73(1): 268.

[25]. Roy R, Kudryashov V, Doty SB, Binderman I, Boskey AL. Differentiation and mineralization of murine mesenchymal C3H10T1/2 cells in micromass culture. Differentiation 2010; 79(4-5): 211-17.

[26]. Martínez-Nava G, Mendoza SL, Fernández-Torres J, Zamudio-Cuevas Y, Reyes-Hinojosa D, Plata-Rodríguez R, et al. Effect of cadmium on the concentration of essential metals in a human chondrocyte micromass culture. Journal of Trace Elements in Medicine and Biology 2020; 62(1): 126614.

[27]. Schäfer S, Urban K, Gerber M, Dekiff M, Dirksen D, Plate U. Dynamic behavior of different quantities of osteoblasts during formation of micromass cultures. Cytometry 2018; 93(4): 458-63.

[28]. Basiri A, Hashemibeni B, Kazemi M, Valiani A, Aliakbari M, Ghasemi N. Cartilage tissue formation from human adipose-derived stem cells via herbal component (Avocado/soybean unsaponifiables) in scaffold-free culture system. Dental Res J. 2020; 17(1): 54-9.

[29]. Zhang L, Su P, Xu C, Yang J, Yu W, Huang D. Chondrogenic differentiation of human mesenchymal stem cells: a comparison between micromass and pellet culture systems. Biotechnol Lett. 2010; 32(9): 1339-346.