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WHICH FACTOR IS BETTER FOR CARTILAGE TISSUE ENGINEERING FROM HUMAN ADIPOSE-DERIVED STEM CELLS? KARTOGENIN OR AVOCADO SOYBEAN UNSAPONIFIABLE

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ABSTRACT

Background: Replacement of the defective cartilage with cell-based tissue engineering products can propose a durable treatment for osteoarthritis. Nowadays, obtaining the best composition as a growth factor for differentiation of stem cells is an ideal goal of cell-based tissue engineering. The current study focused on investigating the effect of kartogenin (KGN) and Avocado soybean unsaponifiable (ASU) on chondrogenic induction of human adipose-derived stem cells (hADScs). Materials and Methods: stem cells were gathered from the human fat and were cultured in the chondrogenic medium within 2 weeks. Then, cell proliferation and viability were evaluated by MTT technique. Chondrogenic differentiation was assessed by histological and histochemical methods. The expression of specific cartilage genes (SOX9, Aggrecan, and type II Collagen) and hypertrophy marker gene (type X collagen) was measured with real-time PCR. The types II and X Collagens proteins were detected by immunohistochemistry technique. Results: KGN increased the expression of chondrogenic genes such as SOX9, COL II, and AGG significantly ($P<0.05$) in differentiated cells than the other groups. Also, ASU raised the expression of COL II and AGG significantly ($P<0.05$) in differentiated cells compared with the control group. But, the expression of SOX9 in ASU group was no significant comparing to the control. The synthesis of cartilage matrix increased in both groups. ASU decreased the expression Collagen X gene in comparison to KGN. Conclusion: The results suggested that KGN induced chondrogenesis in hADScs better than the ASU, within 14 days. But, ASU reduces the expression of type X collagen better than the KGN in the stem cells.

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Introduction

Osteoarthritis (OA) is a degenerative cartilage disease affecting about one-fifth adults in the united states. Also, the prevalence of OA in women is greater than in the men [1]. Present standards of treatments usually end up to fibrocartilage construction and surgeries, and fail to stop the degenerative progression of OA [2]. Today, substitution of defective cartilage with cell-based tissue engineering productions can inhibit OA development [2]. The cell-based tissue engineering has been promoted significantly from developments in the considering of cell gathering, cell signaling, growth factors, and scaffolds [3]. Compared with other kinds of stem cells, hADSCs are good sources of mesenchymal stem cells with multipotential capacity and self-regeneration property [4]. They differentiate between chondrocytes, adipocytes, osteoblasts, myocytes and other cell types [5]. hADScs have two main benefits compared with other stem cells: hADScs do not have political and ethical issues for the reason that they are derived from autologous fat. Likewise, they are obtained from subcutaneous fat in great amount easily [6]. it was reported that the 3D scaffolds are able to mimic cellular environment in vitro. [7]. These 3D scaffolds which are created by biomaterials and biomedical techniques, make a 3D microenvironment for cell differentiation and proliferation, and they promote and regulate the growth of organs or tissues [8]. In 3D scaffolds, the differentiation lineage of adult stem cells (ASCs) is controlled by the mechanical, chemical, and other features of microenvironment [8]. Many studies have shown that hydrogel can capture the cells and inhibit the cell loss, and it has a high delivery capability [9]. Furthermore, the hydrogel can

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generate a biomimetic condition that is more valuable for cell differentiation [9]. Among the many hydrogels, fibrin that is achieved from blood plasma is the most useful hydrogel. [10]. The valuable features of fibrin are that it lacks the potential risk of allergic reaction and microbial infection [11]. Also, it has been reported that cells enclosed in fibrin produce a lot of collagen [12]. Various internal and external factors have contributed to the chondrogenic differentiation of stem cells [13]. Consequently, finding the best growth factor is the ideal goal in tissue engineering. Currently it has been reported that the heterocyclic drug-like molecule, Kartogenin(KGN) as a chondroprotective and cartilage defects inhibitor, was applied to treatment of mouse knee osteoarthritis(OA) [14]. KGN disrupts the association between FLNA and CBFb and controls the RUNX transcription factors [14, 15]. Nowadays, herbal products can be more harmless than synthetic drugs with less unwanted side effects [16]. Avocado/soybean unsaponifiable [15] are vegetable extracts compound made from avocado and soybean oils own anabolic, anti-catabolic and chondroprotective characteristics [17]. ASU induced chondrogenesis of hADScs from PLGA scaffold [18]. It prevents the failure of cartilage and stimulates cartilage renovation by inhibiting much of molecular signaling associated with OA [19]. ASU stimulates the production of collagen and aggrecan via modulation of NF-kappaB and preventing inflammatory cytokines such as IL1, IL6, IL8, TNF, and PGE2 [20] In the current study, we investigated the effect of KGN in comparison with ASU on chondrogenic differentiation of human adipose-derived stem cell.

Materials and Methods

HADScs isolation and culture

Under the sterilized condition, the obtained fat from plastic surgery was transferred to cell culture lab. Then, the fat tissue was washed three times with PBS, and was digested by 1mg/ml collagenase(type1a) (Sigma−Aldrich) at 37°C for 50 min. The cell suspension was centrifuged (1400RPM) for 5 min, and the supernatant was eliminated. The cells' sediment was cultured in medium containing 10% FBS and 1% antibiotics in standard condition. Every three days, the medium was replaced. At the third passage, and 80% cell confluency, the cells were harvested with 0.25% trypsin EDTA(Sigma−Aldrich) and at a concentration of 1×10^6, cells for chondrogenesis on each scaffold were used [21].

Fibrinogen and thrombin preparation

Fibrinogen and Fresh frozen plasma (FFP) were achieved from the Isfahan Blood Transfusion Organization. The FFP(15ml) was mixed with gluconate calcium(10ml), and for 3 hours at 37°, it was incubated and then centrifuged for 10 min at 2500 RPM. The supernatant was collected as thrombin.

Chondrogenic differentiation

HADSCs at a concentration of 1×10^6 were suspended in fibrinogen, and the amount equal to (350µL) of thrombin was added to them. The fibrin scaffolds in chondrogenic medium included DMEM-high glucose with 50 µg/ml ascorbate 2-phosphate, 1% insulin-transferrin-selenium (Sigma− Aldrich), dexamethasone 10^{-8} M (Sigma−Aldrich), 50 mg/ml BSA, 5µg/ml linoleic acid (Sigma−Aldrich),and 1% penicillin/streptomycin without any growth factor was considered as a control group, and chondrogenic medium added to 100 nmol KGN (Sigma−Aldrich) and 10 µg/ml ASU as treatment groups respectively were cultivated at standard condition (37º, 5% CO_2, 95 % humidity) for 14 days. The media were replaced every 3 days.

Cell viability assay

The viability and proliferation of differentiated cells from fibrin scaffolds were estimated with MTT assay(3-(4,5-dimethyl) thiazol-2-yl-2,5-dimethyltetrazolium bromide) (5mg/ml). The chondrogenic medium was removed and the MTT solution (5mg/ml) was added to each well and incubated at 37°C for 4h. Then the medium was omitted, and intracellular formazan was solubilized by adding 400 µl of DMSO. The absorbance of each well was read at 570 nm by ELISA plate reader (Hiperion MPR4, Germany). This analysis was repeated in triplicate [22].

Evaluation of chondrogenic genes expression by Real time PCR

The expression of cartilage-specific genes (SOX9, COLII and AGG) and chondrocyte hypertrophy gene (COLX) were examined by Real-time PCR technique. BY using Trizol, the scaffolds were degraded, and in accordance with the kit protocol, total RNA was extracted from the samples. Afterwards, a complementary DNA (cDNA) was synthesized by the cDNA Synthesis Kit (Yekta Tajhis Azma, Iran). Real-time PCR was completed by SYBRGreen PCR Master Mix and step one Series Software version 2.6 (Corbett Life Science, Australia). The primers for each gene was designed by the primer3 program. (table 1) [23].

Histological techniques

After day 14, the scaffold-cells were fixed with 10% formal saline for 24 hours. The samples were dehydrated, cleared and embedded in paraffin. The 4 µm Sections were stained with Safranin O (MERCK), toluidine blue (MERCK) and hematoxylin and eosin (H&E; MERCK). By Toluidine blue and Safranin O staining, the glycosaminoglycan deposition in ECM was evaluated. For Safranin O staining, the sections were stained with Wiegert's iron hematoxylin for 15 min, with fast green (MERCK) for 6 min and with 1% safranin O for 6 min. After each staining step, the sections were washed in tap water, and at the end, they were rinsed in absolute alcohol. For toluidine blue staining, the sections were stained with toluidine blue for 5min. For H&E staining, the sections were stained with Harris hematoxylin ((MERCK) for 15min and stained in Eosin-Y (MERCK)) for 3 min [24]. On the basis of the proportion and intensity of the color accumulation, deposition of glycosaminoglycan was evaluated.

Immunohistochemistry Assay

For immunohistochemistry assay, the deparaffinized sections were incubated(15min) in 0/3% H_2O in methanol for blocking the endogenous peroxidase. 8 mg/ml hyaluronidase (Sigma) was applied as antigen retrieval for type II Collagen by incubation for 3 hours at 37°C, and 2 mg/ml hyaluronidase as antigen retrieval was used for type X Collagen (Sigma) for 2 hours. Also, type X Collagen samples were treated by 1 mg/ml Pronase (Sigma). Then the sections were incubated with primary antibodies at 4°C (16 hours). Monoclonal antibodies directed against human antigens accessible for type II Collagen (ab3092; Abcam) (diluted 1/50) and type X Collagen (C7974; Sigma) (diluted 1/50). With TBS, the sections were washed and incubated with the anti-mouse IgG secondary antibody (ab2891; Abcam) which was linked to horseradish peroxidase and recognized by using 3, 3′- diaminobenzene (DAB) substrate kit (ab94656; Abcam) [25]. Then, the proportion and intensity of DAB staining were assessed.

Results

MTT assay

The viability of differentiated cells was estimated by MTT assay after the 14th day. Our results indicated that in contrast to control group in which the viability of cells was evaluated near to 98%, cell viability and proliferation in treatments groups, KGN and ASU respectively, had no significant decrease (P>0.05) (Diagram 2). These results showed that both KGN and ASU have no toxic effects on cell viability and proliferation.

Real-time PCR

The analysis of the Real-time PCR results revealed that expression of AGG and COL2 in KGN is significant comparing to ASU and control group (P<0.05). Likewise, the expression of AGG and COL2 in ASU is higher than the control group significantly (P<0.05), but expression of SOX9 in KGN group is higher than ASU and control groups significantly(P<0.05). Although ASU increased SOX9 expression, but it does not significant comparing to the control group(P>0.05). Also the hypertrophic marker gene expression (type X collagen) in both groups isn't significantly higher (P>0.05) than the control group (Diagram 3). In this study, the GAPDH was selected as the reference gene.

Histology and immunohistochemically evaluation

After day 14, by using the histological and histochemical staining, the deposition of GAG in ECM and chondrocyte like cells in lacunas was observed in both treatment groups. This evidence indicated that chondrogenic differentiation of HADSCs in fibrin scaffold happened in both KGN and ASU groups. Toluidine blue and Safranin O stained the glycose amino glycan that can be visualized (Diagram 4-A). But, scoring according to proportion and intensity of color deposition, indicated that the GAG accumulation was higher in Kartogenin and ASU than in control group (Diagram 4-B, C). Localization of specific extracellular matrix molecules on peri cellular, type II Collagen that was visible in cell seeded in fibrin scaffolds confirmed that chondrogenic differentiation of hADScs has occurred (Diagram 5-A). Considering DAB staining, the intensity and proportion of type II Collagen in a group that was treated with KGN was higher than ASU and control groups (Diagram 5-B). The Type X Collagen, the marker of hypertrophic cartilage, was identified in the matrix of all groups. But the overexpression of Type X Collagen in the group that was affected by KGN was higher than the ASU and control groups (Diagram 5-C).

Discussion

Currently, cartilage tissue engineering and cell-based treatments were the best options that were identified in regenerative medicine to control the OA progression and treatment of cartilage defects. But this is very important to ensure that this structure maintains the same functional qualities as native cartilage tissue [26]. Suitable Cells, proper scaffolds and efficient growth factors were the main components of tissue engineering [27]. For the first time, in the present study, the effect of the KGN and ASU on chondrogenic induction of human adipose-derived stem cells (hADSc) in fibrin scaffolds was examined. Fibrin was used as a scaffold for chondrogenic induction. Previous studies confirmed that hydrogel scaffolds, for example, fibrin are not toxic [28]. fibrin in comparison with the synthetic scaffolds is able to surround the cell completely, prevents cell damage, and thus has high distribution ability [29]. Cell proliferation and ECM remodeling increased in fibrin scaffold during chondrogenic differentiation [30]. Our evidence suggested that fibrin scaffold was suitable for Chondrogenesis. Using histology, immunohistochemistry and real-time PCR demonstrated that, ASU induced more chondrogenic differentiation from hADSc within 14 days than the control group. But in comparison with KGN, the overexpression of chondrogenic genes was somewhat less. It seems that the duration of 14 days for chondrogenic induction by ASU is a little short, and more time is needed. In an experiment that [31] conducted on ASU, they observed that the effects of ASU on chondrogenesis on day 21 are greater than the 14th day. Maybe ASU shows its impact in the long run. There is no evidence of the molecular mechanism of ASU on chondrogenic induction in stem cells. But many studies have shown that ASU via inhibition of inflammatory molecules such as interleukins and TNFα increased expression of COL II, AGG and SOX9 from chondrocytes in joint cartilage [19]. Also, previous studies reported that ASU increased the levels of growth factors such as TGF-β1 and TGF-β2 in joint cartilage of canine [32]. It seems that maybe ASU by TGF-β signaling induced chondrogenic differentiation in hADSc but in a long time. Our results revealed that overexpression of the COLX as a hypertrophic marker decreased in the group that was treated by

ASU comparing to KGN group. Many studies showed that enhancement of COLX was an index of chondrocyte hypertrophy that caused a failure in the process of chondrogenic induction [33]. Our results showed that the inhibition of COL 10 gene expression induced by ASU was the most important effect. It was reported that medium containing ASU and TGF- β3 decreased COL10 gene expression significantly by [31], which was similar to our results. There is no report on the effect of KGN on chondrogenic differentiation of hADSCs in fibrin scaffolds. Results of the present study have revealed that KGN can induce chondrogenic differentiation and raise deposition of glycosaminoglycan in HADSc cultured with 100 NM concentrations (Diagram 3). It has been reported that KGN induced chondrogenic differentiation in rat tendons [34]. KGN increased accumulation of glycosaminoglycan in tendon stem/progenitor cells (TSCs) culture and started chondrogenic differentiation [34]. KGN, drug-like small molecule stimulated Chondrogenesis in MSCs [35]. [35] reported that KGN upregulated expression of the specific cartilage genes, and increased the aggrecan and type II collagen protein in MSC. KGN blocked the interaction between CBFb and FC-1 and activated the Runt-related transcription factor X 1 (RUNX1) [15]. This mechanism was identified in the organogenesis period in skeletal precursor stem cells [36]. It seems that KGN, via the RUNX1 pathway shows a key role in Chondrogenesis and chondrocyte proliferation. Upregulation of collagen II gene and enhancement the protein of collagen II indicated that KGN was similar to other growth factors, for example, TGF betas, BMPs or IGFs, and is capable to stimulate the chondrogenic induction in HADSc. Earlier studies revealed that phosphorylation of Smad 2/3 and Smad 1/5/8 is affected by TGF-β family [37, 38]. Likewise, numerous studies have indicated that activation of the Smad 1/5/8 pathway performing upstream of RUNX2 is the basis of hypertrophy and terminal differentiation of chondrocytes [38]. Perhaps KGN only enhances the expression of Smad 2/3 and inhibits the expression of Smad 1/5/8 [38]. KGN by shifting the differentiation balance from late differentiation (hypertrophy) towards early differentiation (Chondrogenesis) may support the articular cartilage. Despite many studies that reported the effect of KGN in chondrogenic differentiation, some studies reported that KGN dose does not induce chondrogenesis, but KGN along with other growth factors is effective [39]. However current study indicated KGN as an inductive factor inducing chondrogenic differentiation in human adipose-derived stem cells.

Conclusion

Our investigation demonstrated that Kartogenin could promote chondrogenic differentiation of hADScs and enhance the accumulation of glycosaminoglycan better than the ASU within 14 days. But, compared with Kartogenin, Avocado soybean unsaponifiable declined expression of the type X Collagen. Our results showed that Kartogenin and Avocado soybean unsaponifiable can be used as inducing factors in the field of cartilage tissue engineering.

Abbreviations

KGN: Kartogenin; ASU: Avocado/soybean unsaponifiable ;TGFβ3: transforming growth factor beta3; ADSCs: adipose-derived stem cells; MTT: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; SOX9: sex determining region Y-box 9; COLII : Collagen type II;COLX: Collagen type X;AGG: Aggrecan; MSCs: mesenchymal stem cells; hMSCs: Human Mesenchymal Stem Cells; N-CAM: Neural Cell Adhesion Molecule; nmol: Nano mole; RT-PCR: Reverse transcriptase-polymerase chain reaction; mRNA: Messenger Ribonucleic Acid; FLNA: Filamin A; CBFb: Core-Binding Factor, Beta; RUNX: Runt-related protein; PBS: Phosphate Buffered Saline; FBS: Fetal bovine serum; DMEM: Dulbecco's Modified Eagle's Medium; FFP: Fresh frozen plasma; BSA: Bovine Serum Albumin; cDNA: complementary DNA; H&E: Hematoxylin and eosin; IHC: Immunohistochemistry.

References

1. Allen KD, Golightly YM. Epidemiology of osteoarthritis: state of the evidence. Current opinion in rheumatology. 2015;27(3):276.
2. Huey DJ, Hu JC, Athanasiou KA. Unlike bone, cartilage regeneration remains elusive. Science. 2012;338(6109):917-21.
3. Smith BD, Grande DA. The current state of scaffolds for musculoskeletal regenerative applications. Nature Reviews Rheumatology. 2015;11(4):213-22.
4. Halvorsen Y-DC, Bond A, Sen A, Franklin DM, Lea-Currie YR, Sujkowski D, et al. Thiazolidinediones and glucocorticoids synergistically induce differentiation of human adipose tissue stromal cells: biochemical, cellular, and molecular analysis. Metabolism. 2001;50(4):407-13.
5. Lindroos B, Suuronen R, Miettinen S. The potential of adipose stem cells in regenerative medicine. Stem Cell Reviews and Reports. 2011;7(2):269-91.
6. Baer PC, Geiger H. Adipose-derived mesenchymal stromal/stem cells: tissue localization, characterization, and heterogeneity. Stem cells international. 2012;2012.
7. Landers R, Pfister A, Hübner U, John H, Schmelzeisen R, Mülhaupt R. Fabrication of soft tissue engineering scaffolds by means of rapid prototyping techniques. Journal of materials science. 2002;37(15):3107-16.

8. Xu Y, Balooch G, Chiou M, Bekerman E, Ritchie RO, Longaker MT. Analysis of the material properties of early chondrogenic differentiated adipose-derived stromal cells (ASC) using an in vitro three-dimensional micromass culture system. Biochemical and biophysical research communications. 2007;359(2):311-6.
9. Taguchi T, Xu L, Kobayashi H, Taniguchi A, Kataoka K, Tanaka J. Encapsulation of chondrocytes in injectable alkali-treated collagen gels prepared using poly (ethylene glycol)-based 4-armed star polymer. Biomaterials. 2005;26(11):1247-52.
10. Andrades P, Asiedu C, Rodriguez C, Goodwin K, McCarn J, Thomas J, editors. Subcutaneous pancreatic islet transplantation using fibrin glue as a carrier. Transplantation proceedings; 2007: Elsevier.
11. Grassl E, Oegema T, Tranquillo R. Fibrin as an alternative biopolymer to Type-I collagen for the fabrication of a media equivalent. Journal of Biomedical Materials Research Part A. 2002;60(4):607-12.
12. Neidert M, Lee E, Oegema T, Tranquillo R. Enhanced fibrin remodeling in vitro with TGF-β1, insulin and plasmin for improved tissue-equivalents. Biomaterials. 2002;23(17):3717-31.
13. Danišovič Ľ, Varga I, Polák Š. Growth factors and chondrogenic differentiation of mesenchymal stem cells. Tissue and Cell. 2012;44(2):69-73.
14. Johnson K, Zhu S, Tremblay MS, Payette JN, Wang J, Bouchez LC, et al. A stem cell-based approach to cartilage repair. Science (New York, NY). 2012;336(6082):717-21.
15. Yoshida N, Ogata T, Tanabe K, Li S, Nakazato M, Kohu K, et al. Filamin A-bound PEBP2β/CBFβ is retained in the cytoplasm and prevented from functioning as a partner of the Runx1 transcription factor. Molecular and cellular biology. 2005;25(3):1003-12.
16. Zhang J, Onakpoya IJ, Posadzki P, Eddouks M. The safety of herbal medicine: from prejudice to evidence. Evidence-Based Complementary and Alternative Medicine. 2015;2015.
17. Lippiello L, Nardo JV, Harlan R, Chiou T. Metabolic effects of avocado/soy unsaponifiables on articular chondrocytes. Evidence-based Complementary and Alternative Medicine. 2008;5(2):191-7.
18. Sharifian Z, Mardani M, Valiani A, Bahramian H, Zamani M, Tavakoli E, et al. Cartilage Tissue Engineering via Avocado/Soybean Unsaponifible and Human Adipose Derived Stem Cells on Poly (lactide-co–glycolide)/Hyaluronic acid composite scaffold.
19. Gabay O, Gosset M, Levy A, Salvat C, Sanchez C, Pigenet A, et al. Stress-induced signaling pathways in hyalin chondrocytes: inhibition by Avocado–Soybean Unsaponifiables (ASU). Osteoarthritis and cartilage. 2008;16(3):373-84.
20. Henrotin Y. Avocado/soybean unsaponifiable (ASU) to treat osteoarthritis: a clarification. Osteoarthritis and Cartilage. 2008;16(9):1118-9.
21. Mardani M, Hashemibeni B, Ansar MM, Esfahani Z, Hamid S, Kazemi M, et al. Comparison between chondrogenic markers of differentiated chondrocytes from adipose derived stem cells and articular chondrocytes in vitro. Iranian journal of basic medical sciences. 2013;16(6):763-71.
22. Esfandiari E, Roshankhah S, Mardani M, Hashemibeni B, Naghsh E, Kazemi M, et al. The effect of high frequency electric field on enhancement of chondrogenesis in human adipose-derived stem cells. Iranian journal of basic medical sciences. 2014;17(8):571.
23. 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. International journal of preventive medicine. 2014;5(7):825.
24. Talakoob S, Joghataei MT, Parivar K, Bananej M, Sanadgol N. Capability of Cartilage Extract to In Vitro Differentiation of Rat Mesenchymal Stem Cells (MSCs) to Chondrocyte Lineage. International journal of molecular and cellular medicine. 2015;4(1):9.
25. Kabiri A, Esfandiari E, Esmaeili A, Hashemibeni B, Pourazar A, Mardani M. Platelet-rich plasma application in chondrogenesis. Advanced biomedical research. 2014;3.
26. Varga I, Zamborský R, Böhmer D. The tissue engineering of articular cartilage: cells, scaffolds and stimulating factors. Experimental biology and medicine. 2012;237(1):10-7.
27. Vinatier C, Guicheux J. Cartilage tissue engineering: From biomaterials and stem cells to osteoarthritis treatments. Annals of physical and rehabilitation medicine. 2016;59(3):139-44.
28. Duong H, Wu B, Tawil B. Modulation of 3D fibrin matrix stiffness by intrinsic fibrinogen–thrombin compositions and by extrinsic cellular activity. Tissue Engineering Part A. 2009;15(7):1865-76.
29. Thomson KS, Dupras SK, Murry CE, Scatena M, Regnier M. Proangiogenic microtemplated fibrin scaffolds containing aprotinin promote improved wound healing responses. Angiogenesis. 2014;17(1):195-205.
30. Hidaka C, Goldring MB. Regulatory mechanisms of chondrogenesis and implications for understanding articular cartilage homeostasis. Current Rheumatology Reviews. 2008;4(3):136-47.
31. Didehvar H, Golshan IF, Valiani A, Hashemibeni B, Esmaeeli M. Comparing The Effects of Transforming Growth Factor Beta1 (Tgf-B1) And Piascledine on the Expression of Collagen Ii, X and Aggrecan Genes in Chondrogenesis of Human Adipose-Derived Stem Cells in Fibrin Alginate Composite Scaffold.

32. Altinel L, Saritas ZK, Kose KC, Pamuk K, Aksoy Y, Serteser M. Treatment with unsaponifiable extracts of avocado and soybean increases TGF-β1 and TGF-β2 levels in canine joint fluid. The Tohoku journal of experimental medicine. 2007;211(2):181-6.
33. Jang Y, Jung H, Ju JH. Chondrogenic Differentiation Induction of Adipose-derived Stem Cells by Centrifugal Gravity. JoVE (Journal of Visualized Experiments). 2017(120): e54934-e.
34. Yuan T, Zhang J, Zhao G, Zhou Y, Zhang C-Q, Wang JH. Creating an animal model of tendinopathy by inducing chondrogenic differentiation with kartogenin. PloS one. 2016;11(2): e0148557.
35. Johnson K, Zhu S, Tremblay MS, Payette JN, Wang J, Bouchez LC, et al. A stem cell–based approach to cartilage repair. Science. 2012;336(6082):717-21.
36. Adya N, Castilla L, Liu P, editors. Function of CBFβ/Bro proteins. Seminars in cell & developmental biology; 2000: Elsevier.
37. Wang Y, Belflower RM, Dong YF, Schwarz EM, O'Keefe RJ, Drissi H. Runx1/AML1/Cbfa2 mediates onset of mesenchymal cell differentiation toward chondrogenesis. Journal of Bone and Mineral Research. 2005;20(9):1624-
38. Wang J, Zhou J, Zhang N, Zhang X, Li Q. A heterocyclic molecule kartogenin induces collagen synthesis of human dermal fibroblasts by activating the smad4/smad5 pathway. Biochemical and biophysical research communications. 2014;450(1):568-74.
39. Liu C, Ma X, Li T, Zhang Q. Kartogenin, transforming growth factor-β1 and bone morphogenetic protein-7 coordinately enhance lubricin accumulation in bone-derived mesenchymal stem cells. Cell biology international. 2015;39(9):1026-35.

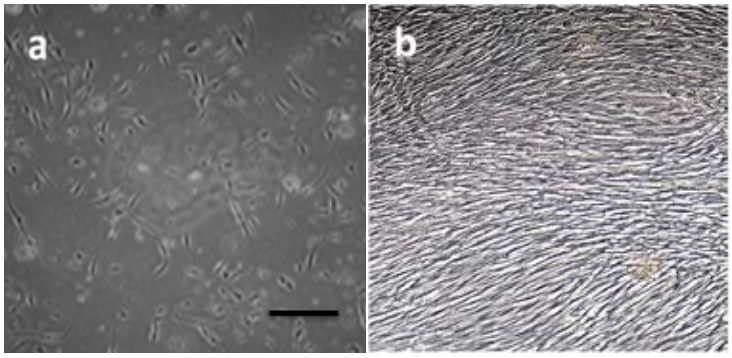


Diagram 1. The image of inverted microscopic of adipose-derived stem cells at passage 3 (magnification ×40), (a: 2nd day, b: 5th day) bar indicated 20 µm.

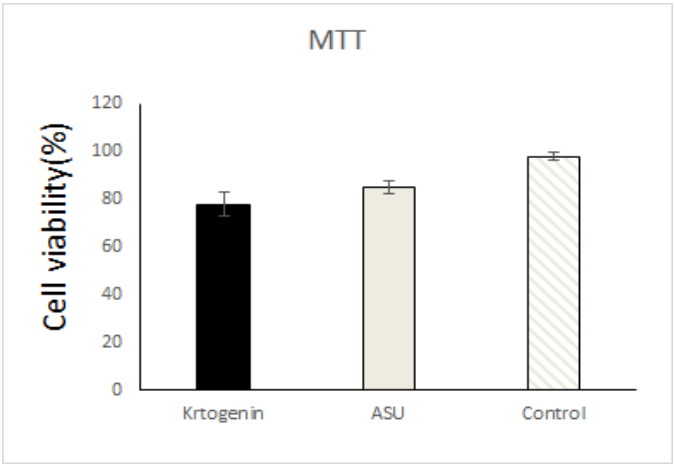


Diagram 2. Comparing the MTT results between the groups on day 14. There is no significant difference between the groups(P> 0.05).

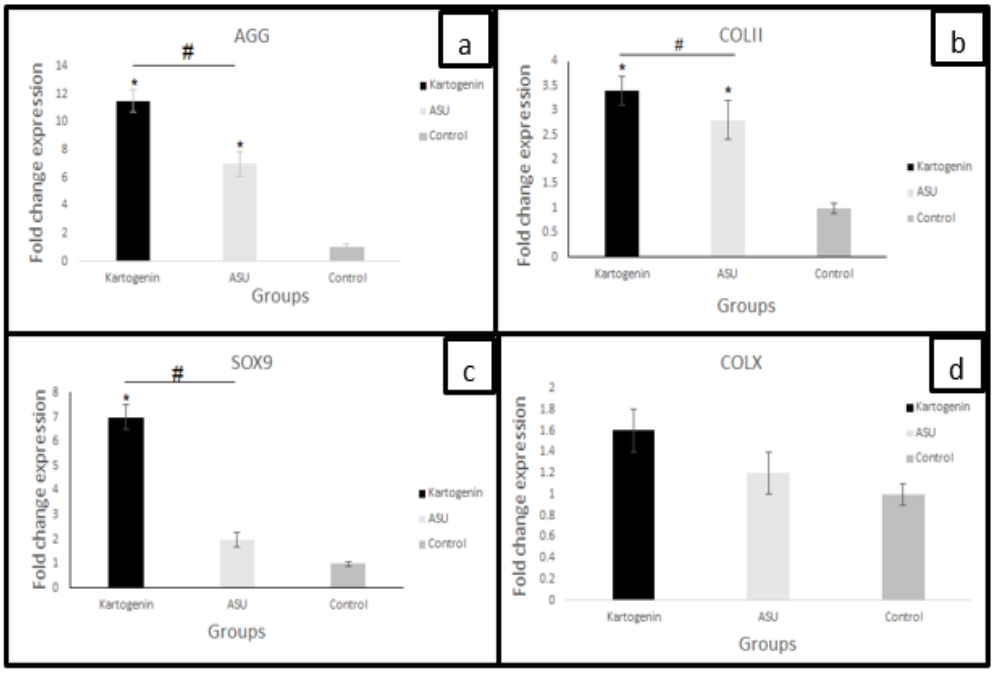
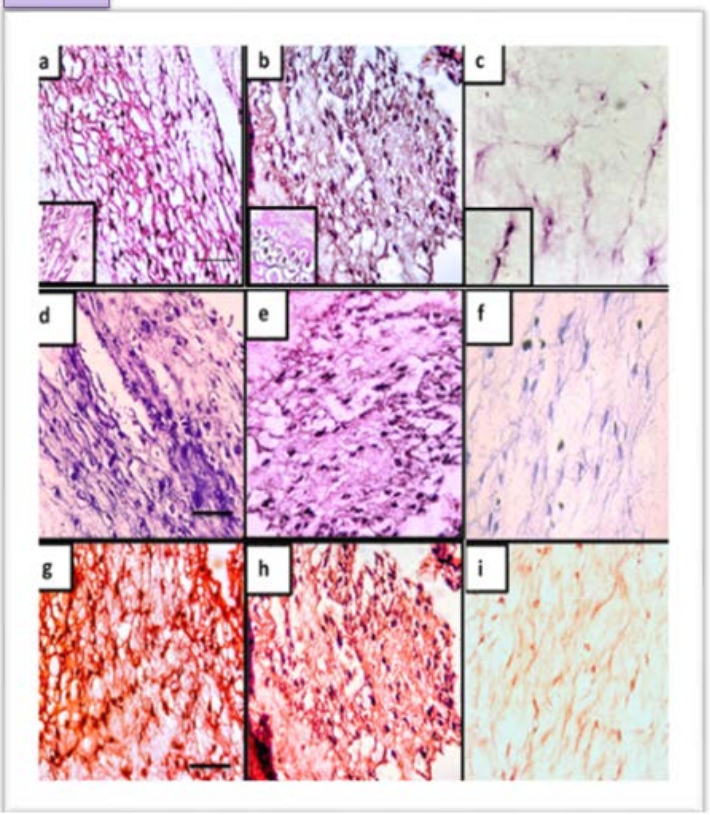


Diagram 3. RT-PCR reaction at day14 for different genes. Data showed as fold changes from day1. a) AGG, b) type II Collagen, c) SOX9 and d) type X Collagen.The Asterisk indicates the significant different from control by (*P<0.05) and the Hash indicates the significant different from ASU by (P<0.05).

A

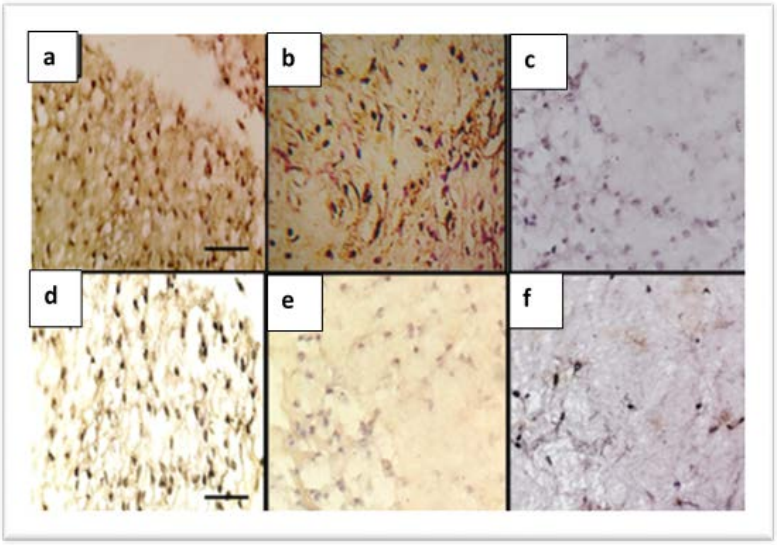


B	Toluidine Blue Staining		
	Proportion	Intensity	Total Score
KGN	9	8	9+8
ASU	6	5	6+5
Control	1	1	1+1

C	Safranin O Staining		
	Proportion	Intensity	Total Score
KGN	8	7	8+7
ASU	5	4	5+4
Control	1	1	1+1

Diagram 4. A) histological sections of neo-cartilage formed by Katrogenin and ASU in fibrin scaffold cultures after 14 days as determined by H&E (a: KGN, b: ASU, C: Control), Toluidine blue (d: Kartogenin, e: ASU, f: Control), Safranin O (g: Kartogenin, h: ASU, i: Control), magnification×40, bar indicated 20µm. B) Scoring of Proportion and intensity of GAG deposition accordance toluidine blue staining (1-10). C) Scoring of Proportion and intensity of GAG deposition accordance safranin o staining. (1-10).

A



B	IHC staining for COLII		
	Proportion	Intensity	Total Score
KGN	8	7	8+7
ASU	3	2	3+2
Control	1	1	1+1

C	IHC staining for COLX		
	Proportion	Intensity	Total Score
KGN	3	2	3+2
ASU	2	1	2+1
Control	1	1	1+1

Diagram 5. A) Immunohistochemically sections of neo-cartilage formed by KGN and ASU in fibrin scaffold cultures after14 days. immunostained with anti-type II collagen antibodies (a: Kartogenin, b: ASU and c: Control) and anti-type X collagen antibodies (d: Kartogenin, e: ASU and f: Control), magnification×40, bar indicated 20µm.B) Scoring of Proportion and intensity of COLII deposition accordance IHC staining (1-10). Scoring of Proportion and intensity of COLX deposition accordance IHC staining (1-10)

Table 1. The genes and primer sequences used for real-time PCR

Gene	primer sequences	Size(base pair)
Collagen II-F	CTGGTGATGATGGTGAAG	130
Collagen II-R	CCTGGATAACCTCTGTGA	
Sox-9-F	TCAGCAGCCAATAAGTG	133
Sox-9-R	GTGGAATGTCTTGAAGGTTA	
Aggrecan-F	ATGGCTTCCACCAGTGCG	127
Aggrecan-R	CGGATGCCGTAGGTTCTCA	
Collagen x-F	AGAATCCATCTGAGAATATGC	187
Collagen x-R	CCTCTTACTGCTATACCTTTAC	
GAPDH-F	AAGCTCATTTCCTGGTATG	125
GAPDH-R	CTTCCTCTTGTGCTCTT	