Research Article

Cartilage Tissue Engineering via Avocado/Soybean Unsaponifible and Human Adipose Derived Stem Cells on Poly (lactide-co–glycolide) /Hyaluronic acid composite scaffold

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ABSTRACT:

Background: Growth factors and chemical stimulants have key role in stem cell to chondrocyte differentiation in cartilage tissue engineering, but this agents have adverse effects on cells as well as they are expensive and they have short half time. Today's there is great interest in the application of herbal agent for treatment of diseases. Avocado/soybean unsaponifiable (ASU) with herbal components has chondroprotective, anti-inflammatory and pro-anabolic effects that it causes stimulate of deposition of extracellular matrix in chondrocytes and relief of osteoarthritis. The aim of this study was an investigation of the chondrogenic effect of ASU in human adipose derived stem cells (hADSCs) on PLGA/HA scaffold.

Materials and Methods:
The 3-D scaffold of Poly lactide-co–glycolic acid (PLGA) prepared via solvent/casting leaching method and impregnated with hyaluronic acid to produce composite scaffold. The characterizations of the scaffold, such as surfaces morphology were observed by scanning electron microscopy (SEM) and the degradation behaviour of the composite scaffold were evaluated. hADSCs seeded in PLGA/HA scaffold and cultured in chondrogenic media with and without ASU. The expression of chondrogenic related genes (Sox9, type II collagen, Aggrecan) and hypertrophic marker (type X collagen) were quantified by real time PCR and viability of cells in different groups were assessed by MTT.

Results:
Our results showed that the expression of genes related chondrogenesis markers Sox9 and type II collagen and aggrecan in differentiated cells in the presence of ASU were significantly increased compared with the control groups (P<0.05), on the other hand, type X collagen expression was not significantly increased.

Conclusions:
Our results indicated that ASU could be as an appropriate inducer for chondrogenesis of hADSCs and cartilage tissue engineering.

Keywords: human adipose-derived stem cells, polylactic acid-polyglycolic acid copolymer (PLGA), Hyaluronic acid, Avocado Soybean Unsaponifiable, Chondrogenesis

INTRODUCTION:
Osteoarthritis (OA) is one of the most common diseases in the middle-aging population and it could cause disability of active populations (1-4). On the other hand Injuries of articular
cartilage have been difficult to self-repair due to the absence of blood vessels and its low metabolic activity(2, 5). Furthermore, common treatments have not a definitive effect of this injury, therefore, tissue engineering as an appropriate and alternative approach in articular cartilage repair has development(6). This treatment strategy requires three key factors: Suitable stem cells with a high chondrogenic potential, appropriate biological stimuli and growth factors for stimulating chondrogenesis induction stem cells and suitable biomaterial for fabricating Scaffold. Scaffolds act as a three-dimensional environment similar to the extracellular matrix(7). Studies in the field of chondrogenesis in embryonic period have shown that mesenchymal stem cells in the early stage accumulate mass, to interact with each other and growth factors eventually lead to the formation of cartilage(8). Moreover, in the chondrogenic process maintaining chondrocyte phenotype requires a three-dimensional (3-D) scaffolds(9). Therefore, extensive studies to achieve appropriate growth factors and 3-D scaffolds in the field of cartilage tissue engineering of stem cells were done, the scaffolds that use in cartilage tissue engineering must be biocompatible, biodegradable, nontoxic as well as having desirable mechanical properties(10). Among a number of 3-D scaffolds reported so far, poly (lactide-Co-glycolide) (PLGA) as a biodegradable synthetic polymer has the most application (19, 20), owing to its prominent advantages such as adjustability of degradation rate, good mechanical properties, especially toughness and excellent processability (21). In addition PLGA is among the few synthetic polymers approved for clinical use by the FDA (22) However, PLGA has a hydrophobic surface and lower bioactivity than natural material (23) Moreover, its high interfacial free energy in aqueous solutions can, unfortunately, influence cell, tissue compatibility in the initial stages of contact(24). To conquer on these disadvantages, some studies have used incorporation of natural polymers in PLGA scaffold (20). In order to improve cell affinity of scaffold and cell attachment; polysaccharides were used (25). Among of natural polymers Hyaluronic acid(HA) that is non-sulfated glycosaminoglycan (GAG) (11) it has appropriate properties such as high biocompatibility, biodegradability, tissue hydration, nutrient diffusion, proteoglycan organization, and cell differentiation, therefore HA is obtaining popularity as a natural scaffold in cartilage tissue engineering studies(12). Human adipose derived stem cells (hADSCs) can be differentiated into multiple cell lineages, including chondrocytes. These cells have great in vitro expansion properties and they are potentially an alternative cell source for cartilage transplantation(6(13)), hADSCs have several advantages, including easy accessibility and minimal invasiveness (14, 15). However, the critical point to an application of ADSCs in cartilage tissue engineering are determining the appropriate environment to induce cellular differentiation. The Presence of growth factors and chemical stimuli such as transforming growth factor-β (TGF-β) (4), insulin like growth factor-1(16) and bone morphogenic protein-6 (17, 18) are necessary. However, researchers tested application of mentioned chemical inducers and found that the neocartilage tissues are not similar to native hyaline cartilage due to having more type I & X collagen and lesser type II collagens, As well as these growth factors, are expensive and have short half time (11-13). Therefore, researchers’ effort to find the alternative agent to induction of ADSCs to the production of a cartilage tissue with better quality and lesser costs. Nowadays, there is great interest in the application of herbal agents for cartilage tissue engineering. (14). ASU has composed of unsaponifiable fractions a proportion of one-third avocado and two-third soybean oil. (15, 16) ASU components showed that to have anti-inflammatory and pro-anabolic effect and chondroprotective properties, as well as in vitro studies showed that ASU, can stimulate production collagen and proteoglycans in chondrocytes (17, 18). Furthermore, in vitro studies suggested that ASU could stimulate the expression of TGFβ1 in chondrocytes(19), in vivo studies demonstrated that ASU could increase TGFβ1 and TGFβ2 levels knee
synovial fluid (20) and it also reduces significantly lesions of OA cartilage in the rabbit (21) Although ASU has been used in the treatment of OA, there isn’t information about the effect of in-vitro use of ASU on chondrogenic induction in hADSCs. The aim of this study was to investigate the impact of ASU on chondrogenic differentiation of ADSCs in the PLGA/HA scaffold.

**MATERIALS AND METHODS:**

1.1. Materials

PLGA copolymer (RESOMER® RG 504H, PLGA; 48/52wt% poly (lactide) / poly (glycolide)); inherent viscosity 0.45-0.60 dl/g ([25 °C; 0.1% in chloroform]) were purchased from Resomer Boehringer Ingelheim, Germany. Methylene chloride (CH2Cl2, M=84.93 g/Mol), tetraethlorthosilicate (TEOS: Si (OC2H5)4), triethyl phosphate (TEP: C6H15O4P), calcium nitrate tetra-hydrate (CA (NO3) 2.4H2O), hydrochloride acid (HCl) were purchased from Merck Inc. The sodium chloride (NaCl) extra pure salt, DMEM LG, trypsin EDTA, sodium pyruvate, dexamethasone, ITS, Premix proline, ascorbate -2-phosphate , bovine serum albumin, linoleic acid, insulin, transferring, selenium (ITS), type I collagenase, Hyaluronic acid sodium salt from streptococcus equi, DMSO were purchased from Sigma-Aldrich. Pure powder of Avocado-soybean obtained from perarin pars. Co, FBS was purchased from Invitrogen, penicillin & streptomycin were purchased from Sigma-Aldrich. (22).

Fabrication and characterization of the composite scaffold:

PLGA scaffold has been prepared via Solvent Casting/ Progen Leaching (SC/PL) penetrating method with using methylene chloride, as previously described with few modifications, as reported previously (22). Briefly, polymer/solvent solution (8% w/v concentration of PLGA in methylene chloride) were cast in cylindrical silicon moulds (9 mm in diameter and 3 mm in height) which was filled with sodium chloride salt particles (NaCl) (approximately 180 µm particle sizes) as porogen. Then, the scaffolds were dried at room temperature for 24 h. In order to leach out the NaCl particles, samples were soaked in deionized water for 2 days. During this period, water was renewed three times. Ultimately, the samples were freeze-dried at -80 °C for 48 h in a freeze-dryer (Christ Alpha2_4Ld Plus, Germany) to produce highly porous construct with no solvent remaining in their structures. Composite Scaffolds were prepared by impregnation of PLGA scaffolds in 2% of solution of hyaluronic acid for 24 hrs. (12). Finally, prepared porous composite scaffolds were sterilized with 70% ethanol and ultraviolet irradiation (U.V) before use. In order to calculate the porosity of the PLGA scaffold, liquid replacement method, were used (23, 24).

Measurements of porosity:

In order to calculate the porosity of the PLGA scaffold, liquid replacement Technique, was used as previously described (25).

Scanning Electron Microscopy (SEM).

The surface and internal structure of the porous scaffold was observed via a scanning electron microscopy (JSM-5600, JEOL). Samples prepared using a sharp razor were mounted on metal and coated with a thin layer of platinum under an argon atmosphere using a plasma sputter (Emitech, K575, UK). (26)

In-vitro degradation:

The degradation rate of scaffolds was assessed after preset time periods. Polymer matrix degradation was assessed by measuring changes in water absorption and it’s mass immediately after incubated at 37°C for periods of time. Scaffolds were immersed in 100 ml phosphate buffer saline (pH 7.4) and stirred in a thermostat (SB-302) at 15 rpm and 37°C. After preset time intervals, the samples were retrieved to determine the water absorption and then dried in vacuum until a constant weight was gained in order to determine the weight loss (27).

Water Absorption of the Scaffold:

The water absorption ratios were measured following the procedure described in a previous report (28). Briefly, each scaffold, cut into 1x1x1-cm3 pieces, was weighed (Wi) before immersion in PBS. Then each group of the scaffolds was incubated in PBS and maintained in a humidified incubator at 37°C/5% CO2 for
14 days. The scaffolds were then removed from the PBS, gently blotted with filter paper to remove surface water, and immediately weighed (Ws). The scaffolds were dried to completely remove the water, after which they were weighed (Wd). The water absorption ratios were calculated using the following equation:

Water absorption ratio = \( \frac{W_d - W_t}{W_t} \times 100\% \)

**Weight loss:**

After the films were recovered at the preset time interval, three samples were weighted after water was removed from the films and they were dried in vacuum until a constant weight was obtained. The weight of the dry film was measured (Wt) and compared with its original weight (W0). The weight lost was calculated using the following equation:

Weight loss: \( \frac{W_0 - W_t}{W_t} \times 100\% \)

**Isolation & proliferation of ADSCs:**

After taking a written consent in the operation room, about 30 gr subcutaneous adipose tissue was obtained from three patients, under the sterile condition and according to the Isfahan University of Medical Science, Medical Faculty Ethic Committee Approval transferred to the lab. It was then digested with 0.075% type I collagenase at 37°C for 30 min. The enzyme was inactivated using of DMEM LG (Sigma) containing 10% FBS (Invitrogen). Then the resultant solution was centrifuged (1500 rpm, 7 min) and cell pellet cultured in 75 cm2 flasks with DMEM LG, 10% FBS, 1% penicillin & streptomycin and incubated with 5% CO2, 37°C. The medium was changed every 4 days. When the cells reached 80% confluence, detached with 0.05% trypsin/0.53 mM EDTA (Sigma) and passaged(29). 3\textsuperscript{th} passage cells were seeded in PLGA/HA composite scaffold.

*In vitro chondrogenic differentiation:*

Harvested hADSCs from passage 3, were resuspended in chondrogenic medium (high glucose Dulbecco’s modified Eagle medium, supplemented with 100 µg sodium pyruvate, 10 µg/ml ASU, 100 nM dexamethasone, 1% ITS + Premix, 40 µg/ml proline, 50 µg/ml ascorbate-2-phosphate, and 1% penicillin-streptomycin, bovine serum albumin 0.5 mg/ml, linoleic acid 5 µg/ml) (27).

For loading the cells on PLGA/HA composite scaffold (3×3 mm and 187 µm pore size) that being in 24 well plate scaffold, 2×10\textsuperscript{6} in 200 ml of medium loaded on each scaffold then plate incubated in 37°C and 5% CO\textsubscript{2} for 2 hrs. Then 500 µml of chondrogenic medium was added to each well. The half amount of medium was replaced every 3 days.

**Experimental design:**

The study setup included two groups; a control group consisting of ADSCs in chondrogenic media without ASU, experimental groups, including a chondrogenic medium with 10 µg/ml ASU. The cells were kept in chondrogenic medium up to two weeks.

**In vitro cell viability:**

The viability of ADSCs with and without ASU was assessed by the MTT assay on the 14th day. The cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. MTT assay was applied to evaluate the effect of ASU on the viability of ADSCs in PLGA/HA scaffold by measuring the uptake and reduction of tetrazolium salt to an insoluble formazan dye by cellular enzymes at 14th day. At first the medium of each well was removed and rinsed with PBS and replaced with 400 µl high glucose medium and 40 µl MTT solution and incubated at 37°C .5% CO\textsubscript{2} and for 4 hrs. Then medium was removed and 400 µl DMSO was added to each well and was incubated in dark for 2 hr. At next step 100 µl of the solution in each well was transferred to a 96-well plate and absorption at 570 nm was measured via ELISA reader (Hiperion MPR4, USA). The assays were performed in triplicate.

The cell viability was calculated by this formula:

\[ \text{Cell viability} = \frac{A_{	ext{test}}}{A_{	ext{control}}} \times 100\% \]
RNA extraction and Real-time polymerase chain reaction (PCR):
At first, scaffolds in different groups were washed with PBS and then were digested via trizol reagent (Invitrogen), then total RNA was isolated by RNase minikit (Qiagen). The RNA concentration was determined using biophotometer (Eppendorf). 100 ng of extracted RNA was used to reverse-transcribed to cDNA synthesis kit (Fermentas) according to manufacturers’ instruction.

Relative quantification of expression SOX9, type II, X collagen and aggregan were measured by using Maxima SYBER® RoxqPCR master mix kit (ferments). primer sequences are shown in table 1. The genes were normalized against the reference gene (GAPDH).

Calculation of expression level of each target gene was performed via $2^{ΔΔct}$ as previously described. (28, 29)

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>primer sequences</th>
</tr>
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<tbody>
<tr>
<td>Collagen II-F</td>
<td>CTGGTGATGATGGTGGAAG</td>
</tr>
<tr>
<td>Collagen II-R</td>
<td>CCTGGAACCTCTGTTGA</td>
</tr>
<tr>
<td>Sox-9-F</td>
<td>TCCAGCAGCCAAATAAGTG</td>
</tr>
<tr>
<td>Sox-9-R</td>
<td>GTGGAATGTCTTGAAGGTTA</td>
</tr>
<tr>
<td>Collagen x –F</td>
<td>AGAATCCATCTGAGAATATGC</td>
</tr>
<tr>
<td>Collagen x – R</td>
<td>CCTTTTACTGTATACCTTTAC</td>
</tr>
<tr>
<td>Aggrecan-F</td>
<td>GTGGGACTGAAGTTCTTG</td>
</tr>
<tr>
<td>Aggrecan-R</td>
<td>GTGTGTCATGTCCTGAAGTT</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>AAGCTCATTTCCTGTGATG</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>CTTCCTCTTGTGCTTT</td>
</tr>
</tbody>
</table>

Statistical analysis:
The comparison of MTT results and gene expression between groups, was performed via independent-Samples T Test analyse of variance analysis. Those with P-value of < 0.05, they were considered statistically significant. All data were reported as mean ± SE.

**RESULTS:**

**Fabrication Porous PLGA/HA composite Scaffold:**
The prepared PLGA scaffolds had 80.5% porosity and size of 180±19 µm. The PLGA/HA composite scaffolds fabricated in this study had 86.5% porosity and pore size of 167 ±12.5 µm.

<table>
<thead>
<tr>
<th>Type of Scaffold</th>
<th>Porosity (%)</th>
<th>Pore Size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA</td>
<td>80.5%</td>
<td>180±19 µm</td>
</tr>
<tr>
<td>HA/PLGA</td>
<td>86.5%</td>
<td>167 ±12.5 µm</td>
</tr>
</tbody>
</table>

**Table 2.** Average Porosity and Pore Size of PLGA and HA/PLGA Scaffolds

**Characterizations of porous PLGA/HA composite scaffold:**

**Surface morphology:**
The prepared PLGA scaffolds had 80.5% porosity and pore size 180±19 µm, the PLGA/HA composite scaffolds fabricated in this study had 86.5% porosity and pore size of 167 ±12.5 µm (Table 2). The data in Table 2 indicate that the PLGA/HA scaffolds showed better porosity and pore size than the PLGA pure scaffolds, at result the PLGA/HA scaffold would provide better cell attachment and viability with positive mechanical properties. In particular, the characterization of certain pores and surface shapes of the PLGA/HA scaffold, as shown in Figure 3 with comparison to PLGA pure scaffolds, well represented the three dimensional and porous microstructure of the NaCl particle shape. NaCl particles 180-220 µm in size were employed as a porogen for the development of porous shape of porous
PLGA/HA scaffolds, since that this size range provided the enough space and proper environment for cells viability without significant cell loss. The development of space within the scaffolds allows impregnated HA dispersing without effect on scaffold microstructure. Unlike the PLGA scaffold, SEM demonstrated a stable three-dimensional and interconnected network microstructure within the PLGA/HA scaffold (Figure 3). Based on these results, we suppose that the impregnated HA contributes to the pores and wall surfaces of the scaffolds helping them to support cell attachment and better match the regenerative tissues at the site of implantation.

![Figure 3. SEM micrographs of PLGA and PLGA/HA scaffolds prepared by the solvent casting/salt leaching and penetrating method.](image)

**Water uptake & weight loss:**
Scaffolds were degraded gently during time. As the polymer scaffold degraded, the water was diffused into the pores of the scaffolds. When the scaffold was immersed in the PBS buffer solution, it was weighted at different preset time. Figure 4 and 5 shows that the mass loss and water absorption characterization of the scaffolds during the degradation intervals. The scaffold with HA composition may have higher water uptake, and higher water absorption leads to greater weight loss which results in more rapid degradation. This event may be ascribed to the hydrophilicity of hyaluronic acid, which leads to a higher water uptake. This event is good for the nutrients diffusion into the internal structure of the scaffolds.
**Fig 4.** Water-uptake of the PLGA/HA composite scaffold that were subjected to the hydrolytic degradation.

**Fig 5.** Weight loss of the PLGA/HA composite scaffold that were subjected to the hydrolytic degradation.

**MTT:**
Dark blue formazan crystals are seen in differentiated cells after treatment with MTT solution. Application of ASU decrease proliferation and viability, but the comparison of results showed (figure 6) that they didn't have significant differences ($P \geq 0.05$). As well as a reduction of proliferation in treating group showed that differentiation is higher than the control group.

**Figure 6.** Comparison of MTT assay results between two groups in 14th day. They have no significant differences ($P > 0.05$). C: control group ASU: the group has been treated by Avocado/Soybean Unsaponifiable

**Real-time PCR:**
The results of real-time disclosed that type II collagen and sox9 and aggrecan genes expression in the group having ASU are significantly higher ($P < 0.05$) than the control group.
The result of real-time showed that the mRNA expression of type X collagen (a negative marker for chondrogenesis) was increased in ASU group. But it isn’t significantly as compared to the control group. (figure 7)

**Figure 7.** Reverse transcriptase-polymerase chain reaction for type II & X collagens, aggrecan and SOX9 genes in all groups at 14th Day. Values are Means ±SE of triplicate experiments, Asterisk indicates that the medium condition is significantly different from control by independent sample t-test (*P<0.05)

**DISCUSSION:**
Since cartilage tissue has not blood supply and nerves, so that it has very poor ability to self-repair. So that finding a reliable method for the repair or regeneration of defects articular cartilage is considered (31-34). Tissue engineering as a most promising approach for articular cartilage defects was noted by using 3-D scaffolds seeded with cells and efficient growth factors(35).

Mesenchymal stem cells (MSCs) are attractive sources for possible clinical application due to self-renewing and multi-potent differentiation capacity. In this regard, adipose-derived stem cells (ADSCs) are the optimum source because feasible accesses to them, large number of stem cell can be obtained from the relatively small amount of fat tissue(36), previous studies show that ADSCs also have multi-potential differentiation ability, including chondrogenesis(37).

Chondrogenesis is process that lead to formation of cartilage or endochondral ossification during embryogenesis and skeletal development (38). When chondrogenesis is triggered, aggregation of chondroprogenitor stem cells is earliest stage to formation pre-cartilage condensation(39).

Studies showed that high density of cells and cellular interaction is essential to produce cartilage tissue in the process of chondrogenesis, through 3-D scaffold high density of cell and cellular interaction can be achieved (40-42). As well as scaffolds can promote of biological functions of cells and ECM synthesis(43). It should be noted that extensive researches has been done in the past to induce chondrogenesis used a variety of scaffold that each have their own advantages and disadvantages. Natural scaffolds are also a number of disadvantages such as low mechanical properties and high rate enzymatic host degradation(44, 45). Synthetic polymers, although good mechanical properties, but they are not suitable biocompatibility and low adhesion property for cell attachment(46).

Previous studies showed that PLGA porous scaffolds are very popular synthetic scaffold due to their good mechanical properties and degradation behavior(47). However, they are not bioactivity to support stem cells growth and...
differentiation and maintain the chondrocytic phenotype, with surface modification by a high bioactive agent such natural polymers can greatly enhance their bioactivity and biocompatibility(48, 49), by impregnating the scaffolds with hyaluronic acid, they can better mimic ECM for cells, consequently, adhesion property and chondrogenesis and cartilage formation are enhanced(50, 51). Jeong Eun Song et al. indicates that incorporation of HA in PLGA scaffold can increase production of aggrecan and collagen(12).

Growth factors are necessary for tissue regeneration especially, in cartilage tissue regeneration(52) and one of the purposes of cartilage tissue engineering is to discover a proper alternative stimulating agent to chondrogenic induction of stem cells to synthesize an extracellular matrix to resemble natural hyaline cartilage. In previous studies, several researchers have studied chondrogenesis of ADSCs in different scaffolds and various growth factors. However, there is no information about chondrogenesis of ADSCs via ASU in PLGA/HA scaffolds.

In this study for the first time we have investigated the capability of ASU in inducing differentiation on hADSCs to chondrogenic lineage in PLGA/HA composite scaffold. Our results disclosed that ASU can increase the extracellular matrix (ECM) and up-regulates the expression of chondrogenesis related genes in hADSCs.

Exogenous use of growth factors such as transforming growth factors-β(TGFβs) and BMP-6 were tested to induce chondrogenic differentiation from stem cells(53-55) but this exogenous recombinant growth factors have short half time and expensive(56-58) and specially TGFβs induce apoptosis in terminal chondrogenic differentiation.(59) For example, Yoo et al. showed that the using of TGFβ-1 as a growth factor in chondrogenic process caused induce expression of type II collagen in 4th day. While in the 11th day, stimulates expression type X collagen that marker of hypertrophy (60). Indra wattana et al. showed that BMP-6 cannot alone stimulate chondrogenesis(61) Other research has shown that BMP-6 increases the level of mRNA of type X collagen in monolayer culture of chondrocytes(62).

Due to problems that related to recombinant growth factor and safety and efficiency of recombinant growth factor, there is a restriction for exogenous use of growth factor in clinical treatment.(63) Whereas ASU is composed of natural component that are beneficial effects on OA(64), previous studies showed that ASU has a chondroprotective effect on chondrocytes(65) moreover ASUs has anabolic effects and capable of stimulating endogenous production of TGFβs in chondrocytes(19). On the other hand showed that TGFβ family can stimulate chondrogenesis in ADSCs.

In this current study for the first time we used ASU in chondrogenic media and our finding revealed that there is an up-regulation in the gene expression profile of sox9, collagen type II and aggrecan. Henrotin YE et al. stated that ASU can stimulate the expression of type II collagen in chondrocytes during culture in a monolayer environment(66). Altinel et al. show that ASU treatment caused an increase in TGF-β1 and TGF-β2 levels in the joint fluid when compared to controls(20). This data suggest that ASU probably capable induce endogenous production of TGFβs in ADSCs as a result chondrogenesis induction can occur in them.

Furthermore, the expression of type X collagen, a hypertrophic factor was consider as limitation during differentiation of hADSCs to chondrocytes. (67) in this study, we found that ASU increased expression of collagen X compare with control but this increasing wasn’t significant, Whereas expression level of collagen type 2 and SOX9 which are specific chondrogenic associated genes significantly high.

The result of MTT show that the proliferation rate in cells of ASU groups compared with control, reduce. This data can indicate that proliferation rate in ASU group decrease and differentiation increase.

**CONCLUSION:**

The avocado/soya unsaponifiable (ASU) was found as a proper, effective inducer in the
chondrogenic differentiation of human ADSCs in 3-D PLGA/HA scaffold. More studies must be done on accessing significant chondrogenesis with other herbal components to promote chondrogenic differentiation.

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