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Original Article

Evaluation of Pomegranate Seed Extract and TGF-β3 on Chondrogenic Differentiation of Human Adipose-Derived Stem Cells

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Abstract

Introduction: The repair of the cartilage continues to be a big challenge. Autologous cartilage is the gold standard, but enough sources are not available. The development of stem cells, biomaterials, and bioactive factors has led to cartilage tissue engineering becoming a promising strategy for the regeneration of cartilage defects. In this study, the effect of Transforming Growth Factor Beta 3 (TGF-β3) and Pomegranate Seed Extract (PSE), as chondrogenesis inducers, were evaluated.

Materials and Methods: Human Adipose-Derived Stem Cells (ADSCs) were seeded in alginate scaffolds and cultivated for two weeks in chondrogenic media. Finally, the MTT assay was used to test the effect of TGF-β3 and PSE on the survival of differentiated cells. The mRNA levels of the cartilage-specific markers such as SRY-box9 (*SOX9*), Collagen type II (*COLII*), Collagen type X (*COLX*), and Aggrecan (*ACAN*) were determined by RT-PCR. Also, CD markers were evaluated by flow cytometry.

Results: Using both natural PSE and chemical TGFβ3 inducers simultaneously, had the best results in chondrogenesis by increasing the expression of the *SOX9*, *COLII*, and *ACAN* genes. Furthermore, the flow cytometry analysis indicated that the expression of CD14 marker of differentiated cells significantly increased, although the expression of CD44 marker decreased two weeks post differentiation.

Conclusions: PSE is a suitable inducer and its combined use with TGF-β3 can improve the efficiency of the chondrogenic potential of ADSCs. Our results suggest that PSE may have the potential to be used in tissue engineering as a replacement for TGF-β3.

Keywords: Adipose-Derived Stem Cells (ADSCs), Chondrogenesis, Pomegranate Seed Extract, TGF-β3

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Introduction

Cartilage damage, such as Osteoarthritis (OA), is one of the most severe chronic diseases characterized by articular cartilage degeneration and a major cause of pain and disability in older adults.[1] Since OA is a multifactorial disorder, its treatment remains a problem for decades. There are various approaches to cartilage treatment but none of these have demonstrated reasonable long-term results. As an appropriate method for cartilage repair, tissue engineering depends on several factors like specific cells, scaffolds and biologically active molecules.[2, 3]

Mesenchymal Stem Cells (MSCs), due to their inherent self-renewal properties, simple to isolate and extend, and homing potential at the injury site, seem to be an ideal source for cartilage tissue formation. Adipose Derived Stem Cells (ADSC) are the most common MSCs with strong proliferative and differentiating potential into various types of mesoderm lineage cells, including osteoblasts, chondroblasts and adipocytes.[4,5] The International Society for Cellular Therapy (ISCT) has suggested positive and negative antigens located on the target cell surface to differentiate MSCs from other cells and to be detected by the antibodies. ADSCs expresses certain MSC markers such as CD44 (Hyaluronate receptors), CD73, CD90, and CD105, but are negative for the CD14 (monocytes), CD45 (white blood cells), and HLA-DR (mature cells) markers.[6,7] A research showed that CD14/45 and CD3/19 markers can be used to distinguish differentiated chondrocytes from undifferentiated stem cells during chondrogenesis and before cell transplantation.[8] The expression of surface markers appears to depend on time in culture, as several evidence indicates that the expression of different MSC markers decreases with an increase in the number of passages.[9]

Injectable and natural biomaterial scaffolds such as alginate

plays a major role in tissue repair and regeneration. Some favorable characteristics of alginate include imitation of the extracellular matrix microenvironment, the ability to promote cell proliferation, migration and differentiation, low cytotoxicity, cell compatibility and a gelling potential.[10-12]

Other key factors required for tissue engineering are growth factors, or bioactive materials. The TGF-β3 has been known to increase secretion of ECM in chondrogenic process.[13,14] TGF-β3 signaling is initiated by the binding to its receptor, and activation of intracellular signaling mediators plays an important role in controlling and promoting MSC proliferation and differentiation, inhibiting osteoblast maturation, and regulating CD expression.[15]

Herbal extracts have many health benefits, and native medicinal plants have been used traditionally as a main source of drugs for the treatment of various diseases, including asthma, OA, heart disease, cancer, swollen ankles, tuberculosis, and hypertension.[16] Pomegranate has been used as a medicinal plant for a long time. Its extract was found to increase the antioxidant capacity *in vitro* and *in vivo*. This activity may be related to the presence of various phenolic compounds present in pomegranate.[17] Research has shown that by oral gavage of pomegranate extract cartilage damage reduces, proteoglycans are less affected, and chondrocyte inflammation are reduced.[18]

In this study, the chondrogenic effect of PSE on encapsulated hADSCs in alginate hydrogel was evaluated. Also, the chondrogenic effect of PSE in the presence and absence of TGF was also evaluated. At the end, we determined gene expression and changes in CD14 and CD44 surface markers in human ADSCs and differentiated chondrocyte during chondrogenesis.

Materials and Methods

Isolation and Proliferation of Stem Cells from Adipose Tissue

The Ethics Committee of the University of Medical Sciences Isfahan accepted all approaches (No: IR.MUI.MED.REC. 1398.667). Subcutaneous abdominal fat was obtained from abdominoplasty patients (20-30 years old) and mesenchymal stem cells were isolated and cultured.[19] To sum up, the obtained adipose tissue washed with PBS (Sigma, USA) twice and then digested with 0.075 percent Type I collagenase (Sigma, USA) solution in a standard condition for 30 min. After neutralizing the enzyme activity, the cell solution was centrifuged at 1400 rpm for 8 min. The cellular pellet was suspended in culture medium containing DMEM-LG supplemented with 10% FBS, 1% penicillin and streptomycin (Gibco, USA), and then cultivated at 37 °C, 5% CO_2. The cells were isolated by applying 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA, Sigma) solution after 80% confluency and then sub-cultured for three consecutive passages.

Pomegranate Seed Extract Preparation

Approximately 3 kg of pomegranate fruits were collected from the Najaf Abad region of Isfahan Province, and about 1.5 kg of pomegranate seeds were obtained and the seeds were handled for several days. The dried pomegranate seeds were powdered and dipped in enough amounts of 70% ethanol solvent and placed on a shaker for 4 h. The extract was then filtered through Buchner funnel After 24 h.[20] The liquid extract obtained through the Maceration technique was concentrated by a 45 °C rotary apparatus in several steps at a pace of about 60 rpm for around a quarter of the initial volume before ethanol was completely evaporated. Then, it was frozen and dried with a freeze dryer before the powder was fully softened.[21]

Encapsulation and Culture Procedure in Alginate

After trypsinization, washing, centrifugation and counting, ADSCs (P3-P5) resuspended in 1.2% alginate (Sigma, USA) at 5×10^6 cells/ml.[22] Then the alginate-cell suspension was dropped by a 22-gauge needle into calcium chloride solution (Merck, USA). The suspension was kept for 15 min at room temperature to form alginate beads. After removing the solution and washing the beads with 0.9% saline solution, this procedure was repeated with sodium chloride. At the end, 1.5 ml of high glucose DMEM was added to each well containing alginate beads.

The chondrogenic medium (2 ml) was added to each well containing alginate encapsulated cells with respect to the groups of control (C), TGF-β3 (T), PSE (P) and TGF-β3/ PSE (TP). TGF-β3 (100 µg/ml), PSE (100 µg /ml) and PSE (100 µg/ml) + TGF-β3 (100 µg/ml) were dissolved in the medium consumed for T, P, and TP groups, respectively. Chondrogenic differentiation medium composed of DMEM-high glucose supplemented with 1% antibiotics (Penicillin & streptomycin [Gibco, USA]), 100 nM dexamethasone (Sigma, USA), 50 µg/ml ascorbate-2-phosphate (Sigma), 5 mg/ml ITS Premix (Sigma), 6.25 µg/ml insulin, 5.5 µg/ml transferrin, 10 µg/ml Selenium (Sigma), 0.5 mg/ml BSA (Bovine serum albumin) (Sigma), 5.5 µg/ml Linoleic acid (Sigma), 100 µg/ml TGF-β3 (Sigma) and 100 µg/ml Pomegranate Seed Extract. The medium changed every 3-4 days, and the differentiation time lasted for 14 days in favorable conditions (37 °C, 5% CO_2, 99% humidity).

MTT Assay

The viability of differentiated cells in alginate bead was assessed by the 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl-tetrazolium bromide (MTT) assay on day 14. At first, the medium of each well was removed, rinsed with PBS, and replaced with 400 µl DMEM and 40 µl MTT solution. Then it was incubated at 37 °C, 5% CO_2 for 4 h, so that purple formazan crystals were formed in the alginate beads. The medium was then discarded and 400 of µl DMSO (Sigma) was added to each well and incubated in dark for 2 h. DMSO dissolved the formazan crystals and created a purple color.

Then, 100 µl of the solution transferred to 96-well plate and the Optical Density (OD) of each well was measured at the wavelength of 570 nm with ELISA reader.[23]

Real Time RT-PCR Analysis

Total RNA was isolated from cells using Biofact Total RNA prep Kit (Biofact, Korea) according to the manufacturer's instructions. The RNA samples were treated with DNase I (Cinnaclon) to remove genomic DNA contamination. About 500 nanograms of total RNA was used to synthesize cDNA using Biofact 2X Onestep RT PCR Master Mix kit (Biofact, Korea) and oligo dT primers according to instructions. The used primers sequences are listed in Table 1.[24] The real-time polymerase chain reaction was performed using Biofact 2X Real-time PCR Master Mix (Biofact, Korea) and the StepOne Plus™ Real time PCR (Applied Biosystems). The PCR amplification conditions consisted of 15 min at 95 °C followed by 40 cycles of denaturation step at 95 °C for 15 sec and annealing and extension for 1 min at 60 °C. Melting curve analysis was used to determine specificity of PCR products. *GAPDH* gene was used as an endogenous control. The expression level of each target gene was calculated as $2^{-\Delta\Delta CT}$, as previously described.[23]

Flow Cytometry Technique

Selection of positive and negative stem cell markers was based on the minimal surface marker proposed by ISCT. In order to specify the hADSCs and differentiated cells, single-cell suspensions in PBS in three special flow cytometry tubes for each CD marker (1×10^5 cells/20 µl in each tube) were prepared and stained with 3 µl antibodies against CD14 and CD44 according to the manufacturer's instructions in dark environments at 4 °C for 30 min. Also, non- specific FITC-conjugated IgG was used for isotype control. Then, the cells were washed two times with 0.5% BSA/PBS and were re-suspended in 500 µl PBS. The percentage of fluorescent cells were analyzed by a FACScan flow cytometer.[25]

Table 1. The sequence of primers

Gene Symbol	Primer Name	Sequence (5' to 3')
GAPDH	GAPDH-F	AAGCTCATTTCCTGGTATG
	GAPDH-R	CTTCCTCTTGTGCTCTTG
SOX9	SOX9-F	TTCAGCAGCCAATAAGTG
	SOX9-R	TTCAGCAGCCAATAAGTG
COLII	COLII –F	CTGGTGATGATGGTGAAG
	COLII –R	CCTGGATAACCTCTGTGA
COLX	COLX –F	AGAATCCATCTGAGAATATGC
	COLX –R	CCTCTTACTGCTATACCTTTAC
ACAN	*ACAN* –F	GTGGGACTGAAGTTCTTG
	ACAN –R	GTTGTCATGGTCTGAAGTT

Statistical Analysis

The data were expressed as means ± Standard Error of Mean (SEM) and were analyzed using one-way ANOVA (post-hoc test) with SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA). The *p*-values less than 0.05 were considered statistically significant.

Results

Characterization of hADSCs During Chondrogenic Induction

The isolated stem cells were passaged upon reaching 80 confluences in primary culture (Figure 1A). After three passage ADSCs appeared flat, large, with a spindle shape morphology (Figure 1B). Also, alginate beads were round and the morphology of the hADSCs encapsulated in alginate hydrogels during chondrocyte differentiation were round and irregular in shape compared with the control group (Figure 1C-F).

Cell Viability

In order to evaluate the proliferation rate of ADSCs during chondrogenic differentiation, the MTT technique was used after culture cells in alginate scaffolds in 14 days after cell differentiation (Figure 2). Followed by the treatment with MTT solution, the dark blue formazan crystals were seen in cells seeded in alginate beads, which indicated their metabolic activity. The results of MTT analysis in four different groups showed that survival rate in the treated groups was significantly lower than the control group ($p<0.001$). In addition, the survival rate of the PSE group was lower than the other groups, which was not significant compared to the TGF-β3 group ($p>0.05$).

Real Time RT-PCR Analysis

Real time RT-PCR was used to evaluate the efficiency of TGF-β3 and PSE on hADSCs differentiation to chondrocyte cells after two weeks. In order to access this point, the genes' expression of *SOX9* (Figure 3A), *COLII* (Figure 3B), *AGC* (as the positive control of chondrogenesis) (Figure 3C) and *COLX* (as the negative control of chondrogenesis) (Figure 3D) were evaluated. The expression of *SOX9*, *COLII* and *ACAN* chondrocyte-specific markers was significantly upregulated in treated groups compared to the control group ($p<0.001$). In addition, the level of *COLII* expression in

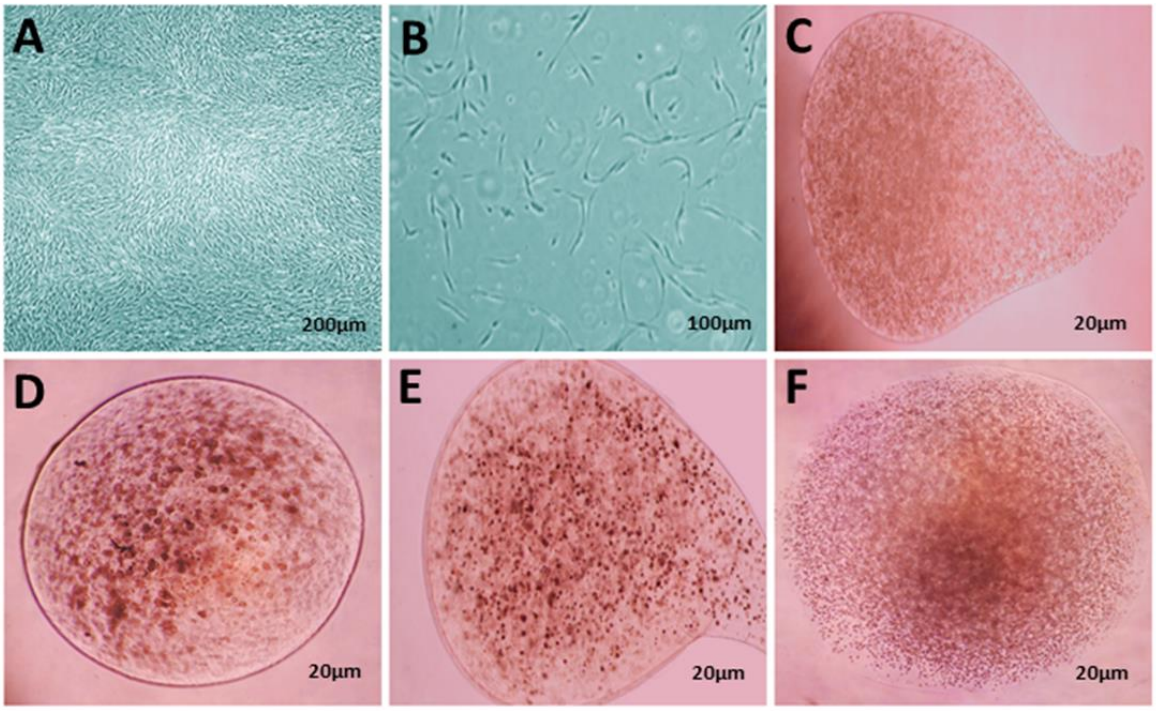

Figure 1. Morphology of ADSCs and Alginate Beads. Inverted microscope images of undifferentiated hADSCs in primary cultured **(A)** and hADSCs achieved a spindle shape morphology after passage 3 **(B)**. Encapsulated hADSCs in alginate hydrogel 14 days' post induction in control group **(C)**, TGF-B3 group **(D)**, Pomegranate seed extract group **(E)** and TGF-β3/ Pomegranate seed extract group **(F)**. Scale bars: A = 200 µm; B = 100 µm; D-F = 20 µm.

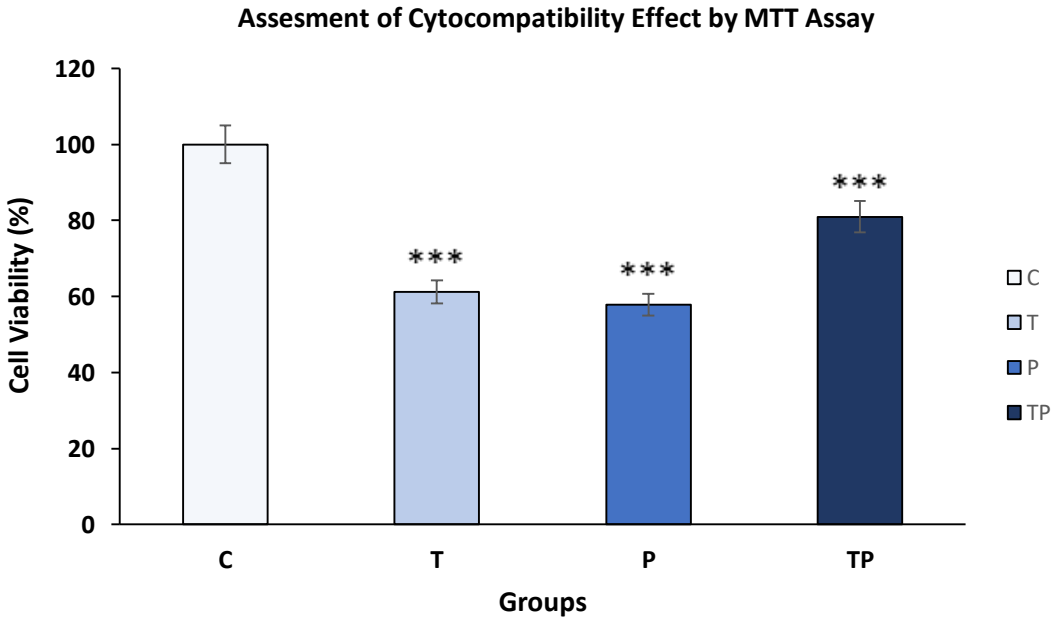

Figure 2. The Cytocompatibility Effect of TGF-β3 **(T)**, Pomegranate Seed Extract **(P)** and TGF-β3/ Pomegranate Seed Extract Group **(TP)** on hADSCs Was Evaluated by MTT Assay on 14 Days. It is noteworthy that T and P groups have negative effect on cell viability and there was significant difference between treated and control groups **(C)** after two weeks' differentiation of ADSCs (***p<0.001 treated groups compared to control group).

differentiated cells was significantly upregulated in the TP group (5.7 ± 0.2) compared to T (4.3 ± 0.2), P (1.5 ± 0.02) and control (1 ± 0.00) groups that showed the synergistic effect of them. Also, the level of *COLX* expression as a negative marker for enhanced chondrogenesis was also significantly reduced in TP (1.5 ± 0.05) and P (1.4 ± 0.05) groups.

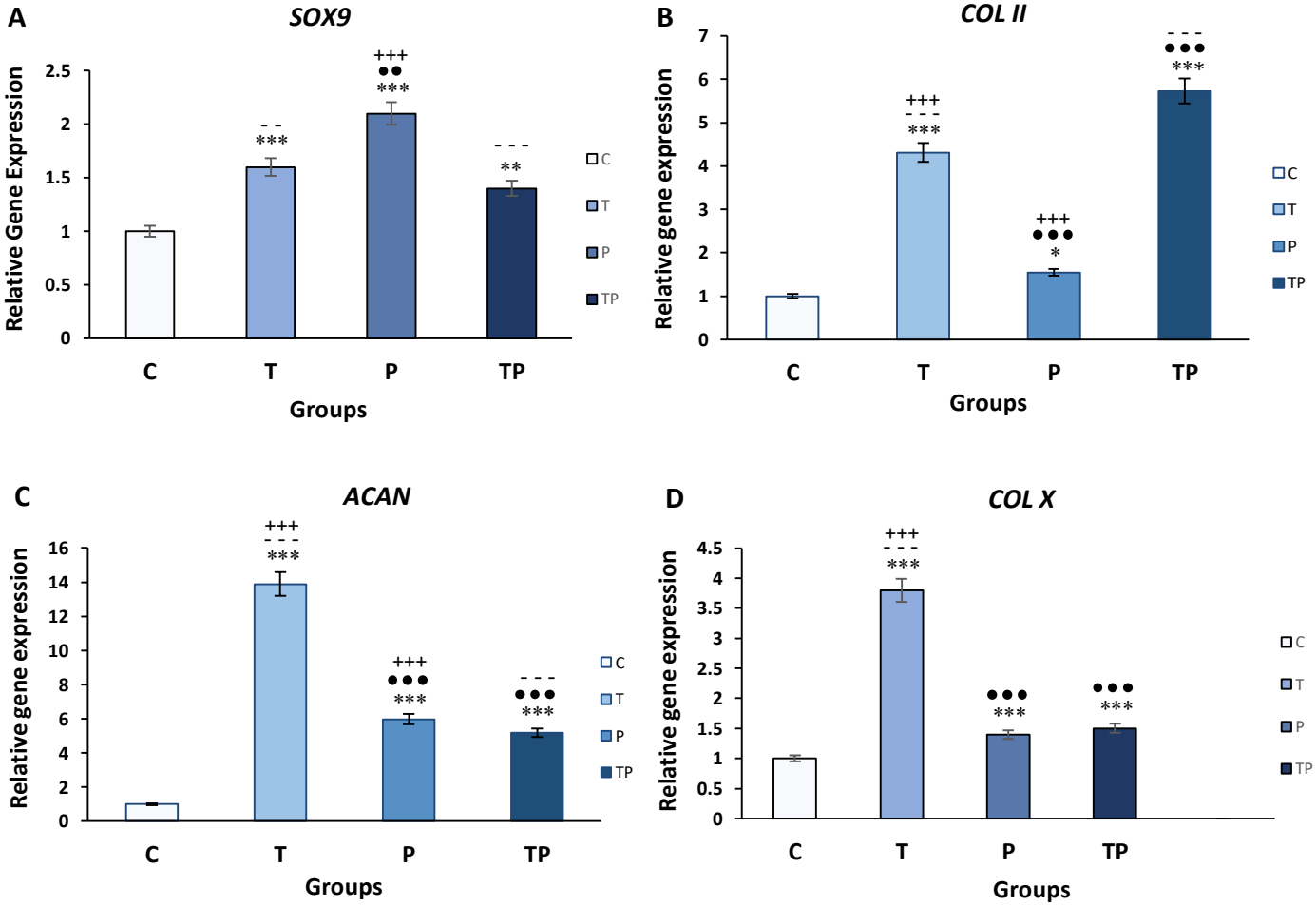

Figure 3. Expression of *SOX9* **(A)**, *COLII* **(B)**, *ACAN* **(C)** and *COLX* **(D)** Genes in Differentiated hADSCs to Chondrocyte Cells by Real Time RT-PCR in Control and Treated Groups. TGF-β3 (T), Pomegranate seed extract (P), TGF-β3/Pomegranate seed extract (TP) and control groups (C) after 14 days. (*p<0.05, **p<0.01, ***p<0.001 indicate significant differences compared to control groups, **p<0.01, ***p<0.001 indicate significant differences compared to T group, - - p<0.01, - - - p<0.001 indicate significant differences compared to P group and +++ p<0.001 compared to TP group). Abbreviations: SRY-related high-mobility-group box 9 (*SOX9*), Collagen Type II (*COLII*), Aggrecan (*ACAN*), Collagen type X (*COLX*).

Flow Cytometry Results

Efficient differentiation of ADSCs to chondrocytes was evaluated by flow cytometry analysis (Figure 4). Flow cytometry analysis of hADSCs within passage 3, revealed that a high percent of these cells were expressed CD44 markers, while a low percent of hADSCs were expressed CD14. Two weeks post differentiation, the percentages of CD44 positive cells was decreased but the percentage of CD14 positive cells was increased.

Discussion

Cartilage destruction was considered as a normal result of the aging process with a small number of treatments options such as drugs and surgery, which are invasive, low-efficiency and are not definitive treatments.[2] Recently the use of stem cells with regenerative potential in various tissues have received much attention.[3] The ADSCs are considered to be an ideal cell source for application in tissue engineering and regenerative therapies, because of several special features such as: simple to replicate from a donor, ability to differentiate into chondrocytes, immunosuppressive properties, low immunegenicity, expressing a number of cell surface markers including; CD90, CD44, CD29, CD105, CD13, CD34, CD73, CD166, CD10, CD49e, CD59, HLA-ABC and STRO-1 and lack of expression of CD31, CD45, CD14, CD11b, CD34, CD19, CD56, CD146 and HLA-DR surface proteins.[5, 26, 27]

Several growth factors are suggested to include in the differentiation and function of chondrocytes. Among these, TGF-β plays a key role in controlling MSC differentiation and cartilage repair via down regulated notch gene expression resulting in increased *SOX9* expression.[28] The *SOX9*, as a master chondrogenic transcription factor, is required for chondrocyte differentiation and expression of several cartilage matrix genes such as *COLII* and *ACAN*.[29] A research showed that TGF-β3 could induce chondrocyte phenotype induction in the high-density culture[30] because, TGF-β3 has no effect on *COLX* reduction. There are however some restrictions,

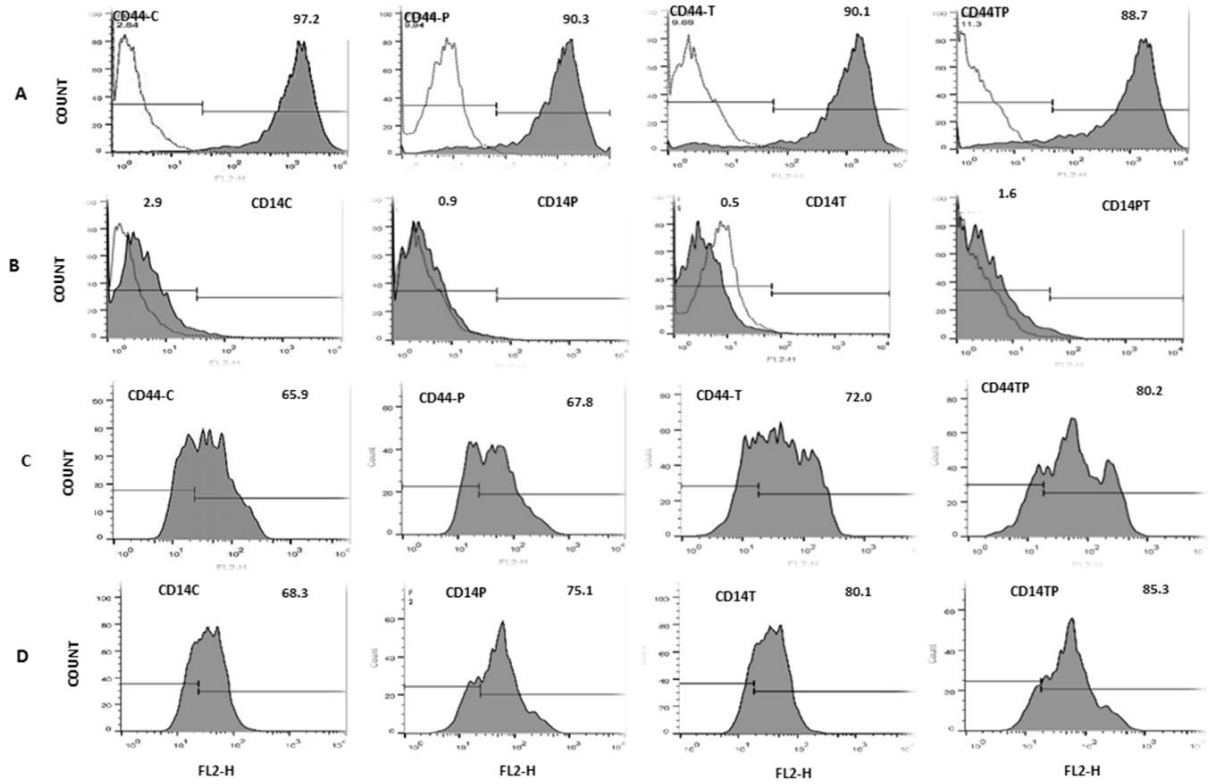

Figure 4. Flow Cytometry Histogram Show the CD Marker Expression Including CD14 and CD44 in Human Derived Stem Cells (hADSCs) **(A, B)** and 14 days' Post Differentiation **(C, D)**. Control group **(C)**, TGF-B3 group **(T)**, Pomegranate seed extract group **(P)** and TGF-B3/Pomegranate seed extract group **(TP)**.

such as hypertrophy and calcification, on the use of this substance.[3]

PSEs are usually described as industrial waste products and have recently received a lot of attention among scientists due to significant levels of bioactive compounds, particularly polyphenols and lipid composition.[31] A previous study has indicated that extracts existing in Pomegranate Seed Oil (PSO) may block the production of the cartilage destroying enzyme. These main mediators of inflammation decrease 75% by seed oil, but pomegranate juice lead to 23.8% reduction in these mediators. Also, an ethanolic extract of pomegranate seeds contain triterpenoids, steroids, glycosides, saponins, tannins, alkaloids, and flavonoids.[32] Monsefi et al., showed the positive effects of pomegranate extract on osteogenesis and chondrogenesis limb bud cultures *in vitro* and in mouse embryos *in vivo*.[33] In a study, the synergistic effect of several pomegranate compounds showed higher value than using a single substance in the suppression of prostate cancer.[34]

In this study we have demonstrated that encapsulated hADSCs in alginate hydrogel when exposed to either TGF-β or PSE could be differentiated into chondrocyte cells. To this end, we evaluated the differentiated cells shape, cells survival, surface markers and some important genes involved in chondrogenesis and hypertrophy. ADSCs were cultured and exposed to 100 µg/ml of PSE, TGF-β3, TGF-β3/PSE and then compared with the control group. ADSCs treated with the medium of chondrogenic induction exhibited a round to oval shape. Differentiated cells survival was analyzed through MTT assay and there was a significant difference between the control group and the treated groups ($p \leq 0.001$). This result suggested that a decrease of cell proliferation by using the differentiation process eventually caused the cells in the treated group to grow much slower than those in the control group.

At each time point, chondrogenesis was evaluated on the basis of mRNA expression of *ACAN*, *COLII*, *COLX* and *SOX9* genes by Real-Time RT-PCR technique and evaluation of protein levels of CD14 and CD44 were measured by flow cytometry in the control and three treated groups. Flow cytometry results showed that although a very low percent of ADSCs expressed CD14, but 14 days after chondrogenic differentiation, the high percent of differentiated cells expressed CD14, especially in treated group with TGF-B3/PSE and the CD44 markers also remained at high percent express but not as the same as the stem cells groups. So, the increase expression of CD14 markers may be related to synergistic effects of TGF-β3 and PSE on the CD14 marker. In this regard, Ghasemi et al., showed that CD14/45 and CD3/19 markers can be used to distinguish differentiated chondrocytes from undifferentiated stem cells during chondrogenesis and before cell transplantation.[8] However, there are some factors

that may be influenced in the degree effects of the PSE on gene expression and surface markers such as concentration of extract, timing, and duration of cultivation.

The present study faced some limitations largely imposed by the study design. One of the limitations was the duration of the design (two weeks). A longer study would provide more information on the potential effect of PSE on chondrogenesis. In addition, evaluation of different concentrations of chondrogenesis induction is necessary to find the most effective concentration with low toxicity on chondrogenesis and cell viability.

Finally, the obtained results are encouraging and prove that the therapy performed using hADSCs encapsulation with both PSE and TGFβ3 inducers simultaneously for chondrogenesis is useful, but more research is still needed.

Conclusion

In this study, we isolated hADSCs and determined cell-surface marker proteins expression in undifferentiated and differentiated cells. In addition, we evaluated the chondrogenic potential of PSE on ADSCs by evaluating the morphology of differentiated cell and mRNA expression of specific-cartilage genes. It was found that PSE, similarly to TGF-β3, had a positive effect on chondrogenic differentiation. Significantly, we observed synergistic effects of PSE and TGF-β3 on the chondrogenic differentiation of ADSCs. These results suggest that PSE may have potential to be used in tissue engineering as a replacement for TGF-β3, although the mechanism of its effects is unknown and needs further evaluation.

Authors' Contributions

All authors contributed equally to the current research.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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