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Comparison of the efficacy of piascledine and transforming growth factor β1 on chondrogenic differentiation of human adipose-derived stem cells in fibrin and fibrin-alginate scaffolds

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ABSTRACT

Objective(s): The aim of this study was to compare the chondrogenic induction potential of Piascledine and TGF-β1 on adipose-derived stem cells (ADSCs) in fibrin and fibrin-alginate scaffolds.
Materials and Methods: Human subcutaneous adipose tissues were harvested from three patients who were scheduled to undergo liposuction. Isolated ADSCs were proliferated in a culture medium. Then, the cells were seeded in fibrin or fibrin-alginate scaffolds and cultured for 14 days in a chondrogenic medium containing Piascledine, TGF-β1, or both. The rate of cell proliferation and survival was evaluated by using MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] assay and the rate of the expression of type II collagen, aggrecan, and type X collagen genes was evaluated by real-time polymerase chain reaction (real-time PCR) method.
Results: The MTT results showed that Piascledine is able to enhance the proliferation and survival of ADSCs in fibrin scaffolds in comparison to other groups (P<0.05). Real-time PCR evaluation revealed that the expression of type II collagen was higher in TGF- β1groups, but the expression of aggrecan was higher in TGF-β1 alone or along with Piascledine in fibrin-alginate scaffolds. Furthermore, the expression of type X collagen was lower in Piascledine alone or along with TGF-β1 in fibrin scaffold.
Conclusion: Piascledine can enhance the proliferation and differentiation of ADSCs in fibrin scaffolds.

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Introduction

Articular cartilage is a special connective tissue composed of chondrocytes and their extracellular matrix. It can be damaged by injury or normal wear and tear. Since articular cartilage is devoid of blood vessels, damaged cartilage takes much longer to heal (1, 2). As a rapidly expanding field, tissue engineering may provide alternative solutions for articular cartilage repair. Tissue engineering uses the combination of cells and suitable biochemicals to prepare appropriate cartilage tissues for the treatment of joint injuries (3).

Different stem cell sources are frequently used in tissue engineering, including bone marrow stem cells (BM-MSCs) and adipose-derived stem cells (ADSCs). BM-MSCs were considered to be the main cellular source of tissue engineering for a good long time but recently ADSCs have received extensive attention due to their availability, less invasive nature and high chondrogenic potential (4).

In addition to using different types of stem cells, various growth factors are also used in tissue engineering. Growth factors play a key role in the proliferation, apoptosis, and differentiation of stem cells. TGF-βs family (transforming growth factor-β) are widely used in cartilage tissue engineering. TGF-βs induce the expression of some genes, like collagen II, glycan and also facilitate the construction of glycosaminoglycans (5). Although TGF-βs have an inducing impact on chondrogenesis of BM-MSCs and ADSCs, they have some limitations that need to be taken into account when using them. Their high price and short half-life (24 to 72 hr) are two of their disadvantages. Besides, these growth factors contribute to the hypertrophy of chondrocytes (6, 7). Piascledine is an herbal supplement that contains the extracts of avocado and soybean in the ratio of 1 to 2. It is used to relieve pain and swelling caused by joint diseases, especially osteoarthritis (8).

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Piascledine has an inhibitory effect on type II collagenase and prostaglandins and a stimulating effect on the synthesis of proteoglycans and collagen. It also reduces fibronectin synthesis and exerting of these effects naturally leads to the restoration of cartilage building. Furthermore, Piascledine inhibits IL-1β and has a stimulatory effect on the synthesis of collagen II in chondrocytes (9) and on TGF-β1 (10). Therefore, it seems that the use of Piascledine alone or in combination with TGF-β1 may help the differentiation of stem cells in culture medium.

Biological materials have a crucial role in tissue engineering. In this regard, a variety of naturally derived and synthetic scaffolds have been used for tissue repair (11). A rewarding scaffold should be biodegradable, biocompatible, and porous. Besides, it must provide suitable conditions for the adhesion, proliferation, and migration of chondrogenic cells (12, 13). A fibrin scaffold is a network of proteins that holds a variety of living tissues together. Fibrin has unique biocompatibility and viscoelasticity properties but its sustainability is weak and degrades rapidly (14). Fibrin scaffold facilitates the cell proliferation and migration, the transfer of molecules and food as well as the disposal of metabolites (15). Several studies suggest that fibrin scaffolds support the proliferation and survival of BM-MSCs (8). The combination of fibrin and alginate increases the sustainability of the resulting scaffold. Alginate is typically extracted from brown algae and its hydrogel form is a porous scaffold. Alginate facilitates the diffusion of macromolecules but at the same time is weak and fragile in terms of elasticity. Therefore, the combination of alginate with fibrin increases its elasticity (14, 16-18).

Some of the major unsolved challenges in the chondrogenesis process are TGF-βs disadvantageous effects and the weakness of scaffolds. The purpose of the present study was to compare the effectiveness of Piascledine, TGF-β1, and the combination of them on the chondrogenic differentiation of human adipose-derived stem cells in fibrin and fibrin alginate scaffolds.

Materials and Methods

Chemicals

All chemicals were supplied by Sigma- Aldrich (St. Louis, MO, USA), unless stated otherwise.

Experimental Design

After the isolation of ADSCs from the subcutaneous adipose tissue and three passages, cells were seeded in fibrin or fibrin-alginate scaffolds. Each of the fibrin and fibrin alginate groups was subdivided into three subgroups: TGFβ1 (10 ng/ml TGFβ1+ chondrogenic medium), Piascledine (10 µg/ml Piascledine + chondrogenic medium) and TGF β1+ Piascledine (10 ng/ml TGF β1+10 µg/ml Piascledine + chondrogenic medium). Triplicate of each subgroup was prepared and cultured for 14 days. On day 14, cells were isolated from fibrin and fibrin alginate scaffolds. Cell proliferation was assessed by MTT assay and cartilage-specific gene expression was evaluated by real-time PCR.

Isolation and culture of human ADSCs

The samples of subcutaneous abdominal adipose tissue were harvested from three people aged 25–40 years who underwent scheduled liposuction. Subjects provided informed consent and the study was approved by the Institutional Ethics Committee of Isfahan University of Medical Sciences. The adipose tissue was digested by collagenase type IA solution at 37 °C for 30 min. Subsequent to that, a complete cell culture medium [DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco)] was added to the cell suspension to neutralize the activity of the enzyme. Then, the cell suspension was centrifuged at 1400 rpm for 7 min and the supernatant was removed along with adipocytes. Finally, the resulting cellular pellet was cultured in a complete cell culture medium at 37 °C, 5% CO_2 conditions (5). Additional cells were removed by changing the medium after 24 hr (Figure 1).

Preparation of Piascledine (herbal blend avocado/ soybean) solution

Piascledine was purchased from Expanscience Laboratories (France). 0.1 mg Piascledine was dissolved in 10 ml of pure ethanol to get to the final concentration of 10 µg/ml.

Thrombin, fibrinogen, and alginate preparation

Fresh frozen plasma (FFP) was used for thrombin preparation. A bag of FFP was obtained from the Blood Bank of Isfahan Province (Isfahan, Iran) and its content melted in a water bath at 37 °C for 10 min. 16 ml of FFP and 10 ml of calcium gluconate were transferred to a sterile tube and then incubated at 37 °C for 60–90 min. After incubation, the tubes were centrifuged at 2200 rpm for 10 min. The clear supernatant of each tube that contained thrombin was aliquoted into 1 ml tubes and was kept at -80 °C (19). To prepare fibrinogen, cryoprecipitate was used. Cryoprecipitate is a satisfying source of fibrinogen.

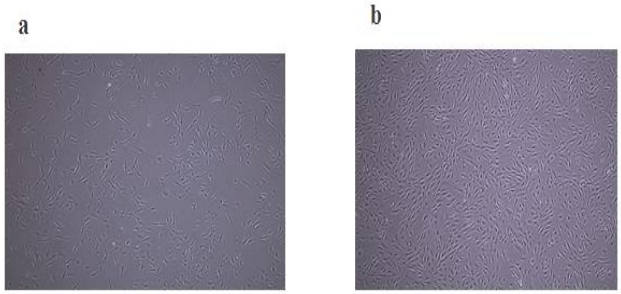

Figure 1. Inverted microscope images of the monolayer culture of ADSCs in the third passage (magnification x40) (a) on the 2nd day (b) on the 6th day

A cryoprecipitate bag was obtained from the Blood Bank of Isfahan Province and placed in a water bath at 37 °C for 10 min. Then, the outer surface of the bag was disinfected with 70% alcohol and its content was removed using a 10 ml syringe under sterile conditions (19).

To prepare alginate, 0.15 g of alginate powder was dissolved in 10 ml of 0.9% sodium chloride and the resulting solution was sterilized by filtration through a 0.2 µm filter (20).

Transferring of cells to fibrin and fibrin alginate scaffolds

300 µl of cryoprecipitate, which contains a large amount of fibrinogen was added to each well of a 24 well cell plate. 30 µl of ADSCs suspension that contains one million cells was added to each well.

Then, 300 µl of thrombin was added and the cell plate was left for 10 min for the formation of the fibrin scaffold (19).

After separation of the third passage of ADSCs cultured cells, the cells were counted. 1 ml of %1.5 alginate was added to every million cells. Then, the cell-alginate suspension was slowly added to 10^2 mM calcium chloride solution (Merck) by passing through a 23 gauge syringe in a 24 well plate. After leaving the solution for 15 min at room temperature alginate beads were formed; then the calcium chloride was removed and the beads were washed three times with 0.9% sodium chloride solution (20). After that, 300 µl fibrinogen and thrombin were added to each well and after a few minutes, fibrin clot containing alginate was formed (Figure 2).

ADSCs were seeded in fibrin or fibrin alginate scaffolds and cultured in chondrogenic differentiation medium [DMEM high glucose (Gibco), 1% Penicillin and streptomycin (Gibco), 10^{-8} mol Dexamethasone (Sigma), 1% ITS (insulin, transferrin, selenium) (Sigma), 1% BSA (bovine serum albumin) (Sigma), 50 µg/ml ASP (ascorbate 2 phosphate) (Sigma), 5 µg/ml Linoleic acid (Sigma)] adding TGFβ1 (10 ng/ml), Piascledine (10 µg/ml) or combination of them (10 ng/ml TGFβ1 and 10 µg/ml Piascledine)].

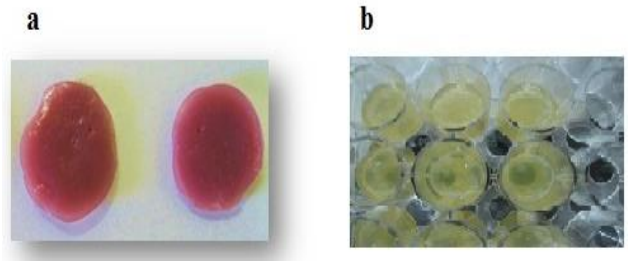

Figure 2. Photographs of chondrocytes seeded in fibrin (a) and fibrin-alginate (b) scaffolds

3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) Assay

Viability tests were applied using MTT colorimetric assay. On day 14, the medium was removed and replaced with 400 µl DMEM high glucose and 40 µl of MTT solution (5 mg/ml in PBS). Then it was incubated at 37 °C, 5 % CO_2 for 4 hr. The medium was then discarded and 400 µl dimethyl sulfoxide (DMSO) was added to each well and incubated for 2 hr in the dark at room temperature. DMSO dissolved the formazan crystals and created a purple color. Finally, 100 µl of each well was transferred to the 96 well plates and the amount of light absorption or optical density (OD) was measured in 570 nm wavelength with an ELISA Reader (Hyperion MPR4). All measurements were done in triplicates (21, 22).

Real-time polymerase chain reaction (Real-Time PCR) analysis

Real-Time PCR was used for the evaluation of gene expression of special cartilage matrix molecules. On day 14, fibrin and fibrin-alginate scaffolds were washed with PBS. For fibrin scaffold, 990 µl of TRIzol with 10 µl of mercaptoethanol was added to the cell mass and the cell plate was placed at room temperature for 5 min. After that, 200 µl of chloroform was added to the solution and after 15 sec of vigorous shaking, the plate was placed at lab temperature for 2–3 min. Then, the solution was centrifuged at 12000 rpm, 4 ºC for 15 min. For fibrin-alginate scaffold, 5.1 mM of 15% sodium citrate (Sharlau) was added to 0.15 mM sodium chloride (Merck) and the solution was centrifuged at 1200 rpm for 10 min to digest alginate beads. RNeasy mini kit (Qiagen, cat. no. 74101) was used for isolation of RNA from resulting cells. The synthesis cDNA was carried out by using Oligo-primers and the revertaid first strand cDNA synthesis kit (Fermentas, England). Real-time PCR was carried out in a *Rotor-Gene 6000* Real-time *Thermal Cycler* (Corbett Research Pty. Ltd., Australia). The PCR mixture contained 10 µl of extracted RNA and 1 µl of oligo, ribonuclease inhibitor, deoxynucleoside triphosphate (dNTP) and Reverstanscriptase enzyme. Then, the following components were added consecutively, 2.5 µl of 10x buffer, 0.5 µl of 10 mM dNTP, 1µl of F Primer, 1µl of R Primer, 2 µl of prepared Taq DNA, 0.5 µl of polymerase enzyme and H_2O to achieve a final volume of 25 µl. Then, cDNA was amplified under the following conditions:

Denaturation at 95 °C for 10 minutes, denaturation at 95 °C for 15 seconds, annealing at 60 °C for one minute and extension at 72 °C for one minute, the whole process was performed for 40 cycles (23-25). In the end, the melting curve was plotted by Melt curve software. This protocol was used for all three genes. All primers used in the real-time PCR were designed by the Allele ID software (ver. 7.6) in accordance with Table 1.

Table 1. Genes and primers used in the real-time polymerase chain reaction

Gene Name	Primer Sequences	Scale
Col II-F	CTGGTGATGATGGTGAAG	0.02 µmol
Col II-F	CCTGGATAACCTCTGTGA	
Agre-F	GTGGGACTGAAGTTCTTG	0.02 µmol
Agre-R	GTTGTCATGGTCTGAAGTT	
GAPDH-F	AAGTCATTTCCTGGTATG	0.02 µmol
GAPDH-R	CTTCCTCTTGTGCTCTTG	
Col X-F	AGAATCCATCTGAGAATATGC	0.02 µmol
Col X-R	AGAATCCATCTGAGAATATGC	

F=Forward primer, R=Reverse primer, Col II= Collagen type II, Col X=Collagen type X, Agre= Aggrecan, GAPDH= Glyceraldehyde 3-phosphate dehydrogenase

Statistical Analysis

All statistical analyses were performed using SPSS software for Windows (Statistical Package for the Social Sciences, version 19, SPSS Inc., Chicago, Illinois, USA). Data were expressed as mean ± SEM. Kolmogorov-Smirnov test was used to check the normal distribution of data.

Data were analyzed using one-way ANOVA and LSD *post hoc* tests. Values with $P<0.05$ were considered statistically significant.

Results

3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) Assay

Our MTT assay results are summarized in Figure 3. These results showed that Piascledine could significantly increase the proliferation and survival of ADSC differentiated cells in the fibrin scaffold in comparison to controls, TGF-β1 alone in fibrin, and TGF-β1along with Piascledine in fibrin groups.

Furthermore, TGF-β1 along with Piascledine increased the proliferation of ADSCs in fibrin scaffold significantly more than TGF- β1 alone in the fibrin-alginate scaffold ($P=0.045$) and also Piascledine alone in the fibrin-alginate scaffold ($P=0.045$).

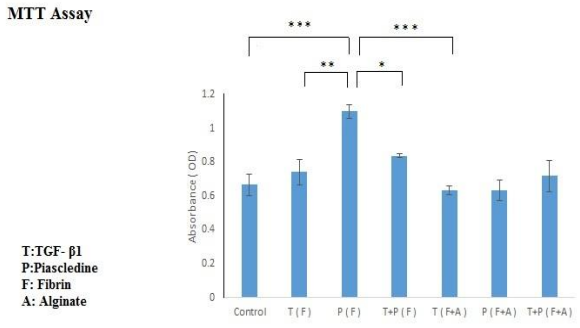

Figure 3. MTT assay results 14 days after the culture of ADSCs in chondrogenic medium supplemented with TGF-β1, Piascledine or both in fibrin or fibrin-alginate scaffolds. Asterisks indicate significant differences between groups. *$P<0.05$, ** $P<0.01$, *** $P<0.001$

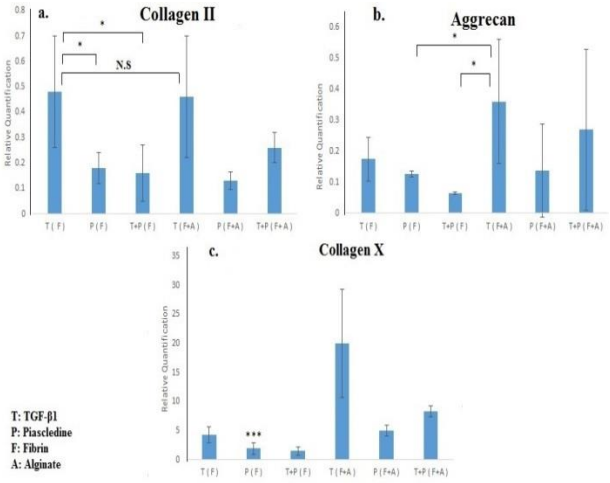

Figure 4. The results of collagen II (a), collagen X (b) and aggrecan genes expression in different groups 14 days after the culture of adipose-derived stem cells. Data are presented as mean ± SD. Error bars represent the standard deviation of the mean. Asterisks indicate significant differences between groups. N.S: Not Significant, *$P<0.05$, ** $P<0.01$, *** $P<0.001$. Relative quantity (RQ) indicates the relative level of gene expression

Real-Time PCR

Real-time PCR results showed significant differences in the expression of collagen II, X, and aggrecan genes in different groups ($P<0.01$). On day 14, the expression of collagen type II gene was significantly higher in TGF-β1 groups (fibrin or fibrin-alginate scaffolds) in comparison to Piascledine groups (fibrin or fibrin-alginate scaffolds) ($P<0.05$). Although the expression of collagen type II and aggrecan genes did not show any significant increase on day 14, Piascledine could induce chondrogenesis alone. The highest expression of aggrecan was in TGF-β1 alone or along with Piascledine in fibrin-alginate scaffold groups. The highest expression of collagen type X gene was in TGF-β1 in fibrin-alginate scaffold group ($P<0.001$). Also, the expression of collagen type X gene is lower in Piascledine alone in fibrin and TGF-β1 alone or along with Piascledine in fibrin scaffold groups (Figure 4).

Discussion

TGFβ1 is a polypeptide member protein that performs many cellular functions such as cell proliferation and differentiation. It is able to stimulate chondrocytes to synthesize type II collagen and proteoglycans. It is also used for inducing chondrogenic differentiation of different mesenchymal stem cells (26). Due to some disadvantageous effects of TGF-βs, such as the generation of hypertrophy in differentiated chondrocytes from ADSCs, other alternatives are considered.

Piascledine (avocado/soybean unsaponifiables) is a natural vegetable extract which is composed of one-third avocado and two-thirds soybean unsaponifiables. At the clinical level, Piascledine reduces pain and stiffness of joints, resulting in decreased dependence on

analgesics (27). It has already been reported that the presence of Piascledine in culture medium for 21 days could induce the chondrogenesis of ADSCs in fibrin or fibrin-alginate scaffolds (28, 29). In the present study, the chondrogenic induction potential of Piascledine and TGFβ1 were simultaneously compared on day 14 post culture of ADSCs in fibrin or fibrin-alginate scaffolds.

Our MTT assay results indicated that the presence of Piascledine is able to enhance the proliferation and survival of differentiating chondrocytes in fibrin scaffolds and it is more effective than TGFβ1. Being consistent with our results, some researchers reported that Piascledine has chondroprotective, anabolic, and anticatabolic properties. Besides, it inhibits the breakdown of cartilage and promotes cartilage repair (8, 9, 30, 31). Also, Piascledine alters growth factor levels implicated in osteoarthritis pathogenesis, increasing TGF-β1 and TGF-β2 in the canine knee joint fluid (32) and reduces inflammation-mediated cartilage degradation by reducing IL-1, PGE2, and MMP-3 production in cultured articular chondrocytes (33).

Piascledine was shown to stimulate collagen and proteoglycan synthesis in cultured chondrocytes (34). An *in vitro* study has shown that Piascledine could stimulate aggrecan production and restore aggrecan production after IL-1 beta treatment (31). In this study, the analysis of real-time PCR results on the 14th day after the culture of ADSCs showed that the expression of collagen type II is higher in TGF-β1 groups. The expression of aggrecan gene was higher in TGF-β1 alone or along with Piascledine in fibrin-alginate scaffolds. Also, the expression of collagen type X gene was lower in Piascledine alone or along with TGF-β1 in fibrin scaffolds. It should be noted that in order to induce chondrogenesis in stem cells and achieve hyaline cartilage, collagen X gene expression should be reduced to prevent hypertrophy of the designed cartilage. Except for the results of collagen type X gene, the obtained results related to collagen type II and aggrecan are in contrary to the previous reports on the 21th day after the culture of differentiating ADSCs. According to these previous results, Piascledine could significantly increase the expression of type II collagen and aggrecan genes in comparison to TGFβ1 during the induction of chondrogenesis in ADSCs (28, 29). It seems that 14 days of culture is not enough time to detect high-level expression of type II collagen and aggrecan genes in differentiated ADSCs and these genes expression will raise 21 days after culture of ADSCs.

Using appropriate scaffolds is one of the main challenges of tissue engineering. These scaffolds should be very similar to the articular cartilage extracellular matrix (5, 35).

In addition, the mechanical stability and inactivity of the scaffold have an important role in the preservation of cells and biomaterials. These biomaterials are necessary for cellular adhesion, proliferation, and differentiation in damaged tissues (36-38). In tissue engineering, biomaterials are specific for each tissue and in most cases, hydrogels such as fibrin, which is composed of the extracellular proteins of the matrix are used (38). A fibrin scaffold has a uniform cell distribution and can easily accommodate biomolecules (15).

This scaffold is biologically degradable and can be used to repair different injured tissues without difficulty (39). Dragoo *et al.* showed that fibrin is the most appropriate scaffold for the recovery of rabbit articular cartilage (40). A study reported that mixing chondroitin sulfate with fibrin increases collagen II and GAG (group-specific antigen) gene expression in comparison to fibrin alone (41). Also, Girandon *et al.* showed that ADSCs are able to reproduce and survive in a fibrin scaffold (42). It is noteworthy that unlike previous studies, which used commercial fibrin, human source fibrin was manually made and used in this study. Although fibrin has unique viscoelastic properties, it is weak in terms of sustainability and also degrades rapidly.

Mixing of fibrin with alginate can increase the sustainability of the scaffold (15, 43). Alginate is obtained from brown algae or polysaccharide capsule of bacteria. Natural polysaccharide alginate easily gels without the need of organic solvents or changing pH and temperature. Although alginate is porous in the gel state and this characteristic facilitates diffusion of macromolecules into it, it is weak and fragile in terms of elasticity. Combination of alginate and fibrin increases its elasticity as a scaffold. In fact, the mixture of fibrin and alginate provides a balance between degradation and elasticity and can create an appropriate extracellular matrix for cell growth. (44-46). Ma *et al.* used the combination of fibrin and alginate as a scaffold to induce chondrogenesis in BMSCs and showed that fibrin enhances the elasticity of scaffold and also increases cell proliferation. Moreover, alginate augments the expression of cartilage-specific genes and extracellular matrix production (47). Our results indicate that Piascledine could significantly support the survival of differentiating ADSCs in fibrin scaffolds better than any other groups. Also, using Piascledine alone or along with TGF-β1 decreases the expression of collagen type X in fibrin scaffolds. The results suggest that fibrin scaffolds provide better results than fibrin-alginate scaffolds.

Conclusion

Piascledine can improve the proliferation and survival of differentiating ADSCs to chondrocytes in fibrin scaffolds. Although previous reports demonstrated that Piascledine can increase the expression of collagen type II and aggrecan after 21 days culture of differentiating ADSCs, 14 days of culture is not enough time to detect high-expression of these genes. Also, using Piascledine decreases the expression of the hypertrophic marker, collagen type X in fibrin scaffolds.

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Conflict of interest

There are no conflicts of interest in this research.

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