Investigation of the Synergist Effect Extract of Licorice and Elaeagnus Angustifolia on the Chondrogenesis of Human Adipose-derived Stem Cells on Fibrin Scaffold

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Keywords: licorice, Elaeagnus Angustifolia, , stem cells, fibrin, scaffold

Posted Date: January 21st, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1269750/v1>

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Abstract

Background: Herbal medicines have recently taken a special place in medical treatment and research. The study was performed to evaluate the effect of *E. angustifolia* L and licorice extract on cartilage repair using ADSC in fibrin scaffold.

Methods: In the study, stem cells were isolated from adipose tissue by mechanical and enzymatic digestion of subcutaneous adipose tissue of humans. In a DMEM-enriched medium, 1% penicillin and streptomycin were cultured in T25 flasks and incubated at 37 ° C and 5% CO₂ pressure. After the third passage, the cells are implanted in fibrin scaffolds. Cells cultured in scaffolding are divided into four groups: 1- control group (medium chondrogenic (DMEM)) 2- DMEM + Lic L (25), 3- DMEM + *E. angustifolia* L (20), and 4 - DMEM + Lic L + *E. angustifolia* L . After 14 days, cell viability was assessed using the trypan blue and the gene expression was measured using the Real Time- PCR method.

Results: The average cell viability in different groups is above 70%, which is acceptable. Examination of COL1A1, AGG and SOX9 gene expression in general showed that the presence of *E. angustifolia* L and Lic L at the specified time was effective on gene expression ($P \leq 0.05$). In the concomitant use of extracts, the relative expression of genes was higher than other groups, but to a lesser extent also caused hypertrophy.

Conclusions: This study confirms that Lic L and *E. angustifolia* L in the presence of fibrin scaffolding could be an effective method in improving cartilage lesions. Concomitant use of extracts has been more effective in gene expression as well as the viability of generation cells compared to other groups. But a small amount also causes hypertrophy as well as the expression of the coll X gene.

Introduction

Renovation of cartilage tissue damage is a significant problem in orthopedics, but more recently, tissue engineering techniques can be an acceptable solution to untangle this example of a problem. (1) The purpose of tissue engineering is to create options that enable the recovery, maintenance, and function of damaged tissue. In tissue engineering, cells are taken from a person, and after culturing and increasing their number, they are planted on scaffolding and new cells are formed in a short time. (2)

Scaffolding plays an essential role in tissue regeneration and repair. (3) Scaffold is a three-dimensional structure used to guide the cell to grow and is an alternative to the extracellular matrix. (1) Cells penetrate the scaffolds and begin to grow, differentiate, multiply, and migrate against the physical and chemical signals of their environment, and if environmental conditions are favorable, they secrete an extracellular matrix. (3) The ideal scaffold should have good porosity for food distribution and good mechanical stability. It also increases cell adhesion and cellular signaling so that cells maintain their natural phenotype. (3) Fibrin, an irresoluble protein that is constructed in reply to bloodshed and is the main ingredient of the blood clot. it is organized from fibrinogen, a solvable protein that is created by the liver

and found in blood plasma. (4) Fibrin is a natural biopolymer with many interesting characteristics. It is biocompatible, easy to process, and well adapted to different polymerization conditions. (5)

Licorice (Lic L) is one of the medicinal plants in Iran that is exported in significant quantities annually. The main ingredient of this species is a combination of a triterpenoid saponin called glycyrrhizic acid or glycyrrhizin, which is used in the pharmaceutical and food industries and has a sweetness of 30 to 50 times that of sucrose. (6) Licorice root contains various compounds such as various sugars, flavonoids, sterols, amino acids, gums and starches, essential oils, and saponins.(7) The compounds of glycyrrhizin plant have anti-inflammatory and antioxidant properties that are used to treat stomach ulcers, rheumatoid arthritis, respiratory diseases, etc. (8)

Elaeagnus Angustifolia fruit(of *E. angustifolia* L) contains significant amounts of flavonoids, terpenoids, phytosterols, carvacrol, glucose, fructose, phenolic acid. In traditional Iranian medicine,(9) elm fruit has been introduced as an analgesic agent in patients with arthritis. (10) Recent pharmacological studies have also shown that this plant has antibacterial, antioxidant, and anti-inflammatory effects.(11)

Different tissues in the body are made up of cells that can produce cells like themselves or adjacent cells, including cells of the heart and nervous system. There are cells in the body that have special capacities to differentiate and become specialized cells. These cells have two distinct characteristics: one is that they can become specialized cells in the body, and second, they can form their stem cells and those around them and remain unchanged during large divisions and long periods.(12)

ADSC also retains its differentiation potential in cells of its origin and is usually characterized by high immunity and modulatory effects. Abundance, accessibility, ease of stem cell isolation as well as differentiation and proliferation of these cells have made this tissue known as an important source for obtaining stem cells. (13) Therefore, the International Federation of Fat Treatment (IFATS) adopted the name ADSC for greater uniformity. (14)

Methods

The method of separating the stem cells of fat and tissue passage(DMEM high glucose (Gibco) Penicillin–streptomycin , Insulin transferrin–selenious sodium 50 µg/ml Ascorbate–2 phosphate (Sigma) Dexamethasone (Sigma) ,Linoleic acid (Sigma) . collagenase(sigma)

Adipose tissue was removed from the patient's abdomen using liposuction and surgery and placed in a sterile container containing 1% antibiotics and culture medium at 4 ° C to prevent cell destruction. Using a razor, the extra blood vessel and connective tissue were removed from the fat and then 0.5 mg per ml of collagenase was added per gram of fat. And incubated for 45 to 60 minutes at 37 ° C to separate stem cells from adipose tissue. Centrifugation was performed for 15 minutes at 2200 RPM. The cells deposit at the end of the Falcon tube, and in the Falcon tube, there are three stages: a layer of adipose tissue and the middle phase of the environment and enzyme, and the third stage is the stem cell. The flasks contain DMEM. , Penicillin and streptomycin. The head of the cow embryo was transferred. It was placed in an

incubator with a pressure of 5% carbon dioxide and a humidity of 95%. After 48 hours, the cells that were free and did not adhere to the end of the flask were removed by changing the culture medium. (figure 1). (15)

1) Cut the fat into small pieces. 2) Add 0.5 mg of collagenase 2 per gram of fat. 3) Place in the incubator for 60 to 90 minutes. 4) Add the same amount of FBS. 5) for 15 minutes at 2200 RPM in a centrifuge. 6) Hold the third phase and remove the rest of the contents of the Falcon tube. 7) Add 6 ml of culture medium. 8) Transfer the cells to the flask. 9) Place in the incubator.

Expansion

After the density of cultured cells in the flask reached above 80-90%, the whole-cell culture medium was drained and washed with 5 ml PBS to remove proteins and disturbing cell components. Then PBS was removed and 2 ml of trypsin / EDTA was added to it and placed in an incubator for 2-3 minutes. Using neo bar slide and trypan blue stain, we counted the number of cells and divided them in a ratio of 1: 3 to be used for the next passage.(16)

differentiation of adipose stem cells into cartilage cells:DMEM(Contains 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate.

The cultured mesenchymal stem cells were isolated from the bottom of the culture flask using trypsin and cultured in a ready-made fibrin scaffold. Cells in certain groups containing compounds (the first group of of Lic L and the second group of of E. angustifolia L and the third group of of E. angustifolia L and Lic L simultaneously and four control group(medium chondrogenic)) and DMEM with insulin and 1% FBS and penicillin and streptomycin ascorbate 2 phosphate store 50 micrograms per liter They were incubated for 14 days at 37 ° C and 5% carbon dioxide pressure and we change the culture medium every three days.

Fibrin scaffold design: The new plasma was prepared in coordination with the blood ward of Shahid Sadoughi Hospital in Yazd. Fresh plasma was mixed with calcium gluconate in a ratio of 5 to 3 and incubated at 37 ° C for 60 to 90 minutes. It was then centrifuged at 2200 rpm for 10 minutes. After doing this, the contents of the Falcon tube form two separate phases, the transparent thrombin layer at the top, which is used by us to make hydrogel scaffolds. (1)

Extraction and Purification of RNA

The use of RNA XPLUS is a common method in most laboratories in Iran. The basis of this method is based on guanidine thiocyanate and phenol-chloroform.

Turn on the centrifuge and place it at 4 ° C.2-Prepare microtube 2 containing 1000 λ RNA XPLUS.3- Prepare the razor and slide and put the ice bucket under the hood.4- Pour the cell and scaffold into RNX PLUS and mix for 15 seconds. Leave at room temperature for 5 minutes.5-Then vertex the microtube containing the homogenized cell and scaffold for 15 seconds and place it for 5 min at room

temperature.6-Add chloroform with 200 λ with the yellow head sampler and make 15 s stylish by hand.7-Incubate on ice for 5min.8-Then place in a centrifuge at 12000 rpm at 4 ° C and Rpm for 15 min.9-150 λ three to 50 λ with the yellow head sampler, remove from the upper phase and transfer to microtube 1.5.10-Add the same fuzzy volume that we took, 150 λ isopropanol, mix 5 s, and put 15 min on ice.11-Place for 15 minutes in a centrifuge at 12,000 rpm and 4 ° C. At this stage, we make 75% alcohol for To make 1 cc of 75% ethanol, we use 750 λ alcohol + 250 λ sterile distilled water and turn on the heater, and put it at 60 degrees Celsius.12-Discard all supernatants (total microtube content) and add 1 cc or λ 1000% 75% alcohol made in the previous step to the microtube and place in a centrifuge at 7500 rpm and 4 ° C for 8min.13-Remove the entire contents of the microtube and place it on sterile gas for 6 minutes.14-Add 35 λ DEPCwater.15-Put the heater at the set temperature for 10 minutes.16-We put 2 λ RNA in the spectrometry .

The sincerity and quality of RNA are determined based on the UV absorption of 260 nm and the ratio of 260 to 280 by the spectrometry device. Expression of Gene IN RT-PCR and Quantitative real-time PCR(SOX9-COLL2-COLL10-AGG)For RealTime-PCR, 1.5 μ l of cDNA was added to SYBR Green qPCR MasterMix 2X which was half the final volume(table 1). The program started at 95 ° C for 15 minutes and continued according to the schedule in the table below. The second program, including Denaturation, Annealing, and Extension, was repeated in 40 cycles, respectively. Finally, the melting curve of the Melt curve (60 ° C $\rightarrow$ 95 ° C) was plotted by step one plus Applied Biosystem. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was applied as an internal control gene. The differential expression of genes was calculated by the $2^{-\Delta\Delta CT}$ method. (Primer sequences are shown in table 2) **Statistical analysis**

one-way ANOVA tests are used to compare quantitative data. Statistical analysis was performed with SPSS software version 16.0 which runs on a computer.

Results

Evaluation of cell viability using trypan blue staining

Examination of cell viability revealed that all groups were significantly different from the control group, but the group in which and Lic L was used was not significantly different from other groups.(Figure 3)

Evaluation of the results of gene expression through RT- PCR technique:

Examination of chondrocyte-specific genes in different groups showed that after 14 days, the cells were in the path of chondrogenesis induction. Gene expression in different groups was compared with the control group.

Investigation expression of gene:

Examination of gene expression, in general, showed that in the specified period, the presence of E. angustifolia L and Lic L affected gene expression (P-value ≤ 0.05). The expression level of SOX9 gene

was significantly higher in the group that received the extracts at the same time than the other groups was 28 times higher than the control group. Also, the extracts alone were effective in gene expression. Agrecan gene expression was 17.3 times higher in the group that used the combination of licorice extract and *Elaeagnus angustifolia* extract than in the control group. The expression level of type II collagen gene in a given period was significantly higher in the group that received *Elaeagnus Angustifolia* fruit extract and licorice than other groups. The expression of type X collagen gene in the combination group of licorice extract and *Elaeagnus Angustifolia* was 4.8 times higher than the control group.(Figure 5)

Discussion

Hyaline cartilage tissue lacks any blood supply and therefore the associated lesions are almost difficult to repair; On the other hand, the treatment method that is commonly used to repair these lesions is not effective. From this, tissue engineering and restorative medicine have expanded as an alternative method in recovering lost tissues in the last decade. Mesenchymal stem cells are considered an ideal source for use in tissue engineering due to their ability to proliferate and differentiate into several cell lines. Since normal articular cartilage typically has low cell density and does not repair spontaneously, (17, 18), sufficient cells with high bioavailability must be transplanted in the area of cell lesions. ADSC is considered an ideal source due to its easy availability and the number of stem cells obtained each time adipose tissue is extracted. In this study, it was used as a cellular source to prepare cartilage tissue. One of the important factors and features in cartilage tissue engineering is the preparation of scaffolds that can mimic or resemble the extracellular matrix and connect well with the host tissue. (19, 20) Studies have shown that such a density of cells that interact with each other should use three-dimensional culture media (scaffolds) through which to achieve a high density of cells in an environment. In addition, scaffolds are effective in increasing the biological function of cells and producing an extracellular matrix (129). So far, extensive studies have been used on the use of a wide range of biomaterials as scaffolds, each with its advantages and disadvantages. In general, there are two types of scaffolding: natural scaffolding such as fibrin, collagen, alginate, hyaluronic acid ... and synthetic scaffolding such as polylactide acid, polyethylene glycol, co-polymer polylactide glycolic acid.

Fibrin scaffolds can be used as effective scaffolds because of their ability to help cells differentiate and proliferate, but the main problem with these scaffolds is their poor stability. In the study, fibrin hydrogel was effective in cell survival, proliferation, and differentiation. Although fibrin gel has been introduced as a sponge, it can be used as a three-dimensional platform for cartilage repair.

Hamidpour et. al(2017) intending to investigate Elm (*Elaeagnus Angustifolia* L.) from a variety of traditional drug programs to its new roles as active antioxidants, anti-inflammatory, anti-mutant, and analgesic, as well as In wound healing, the findings of the study indicate that after the use of *E. Angustifolia* L. fruit extract in the wound area, the content of hydroxyproline, an important factor in collagen production, increased in the tissue. This is in line with our research that using licorice and elm extract simultaneously and separately, the expression of collagen genes has increased compared to the control group, which did not find any effective substances.(21)

SoheilaHamidpour and Rafi Hamidpour et. al(2019) with the aim of chemistry, pharmacy, and medicine of Russian olive or *ElaeagnusAngustifolia* L, it has been determined that the compounds of sesame extract or olive Russian cause the production of proline and hydroxyproline, which are the main components of collagen, which improves wounds and anti-inflammatory effects. Which is observed simultaneously or separately with the results of real-time tests related to licorice and elm extracts. According to the study, these extracts cause the expression of collagen genes.(22)

Goodarzi et.al(2017) For the protective effect of *ElaeagnusAngustifolia*L. Alcohol, the fruit of the hydroalcoholic extract of nephrotic toxicity caused by cyclophosphamide in mice, had the highest effect between doses of 20 to 40 mg. We also used 20 microliters.(23)

Do Seol et. al(2012) to examine the response of chondrogenic progenitor cells to cartilage damage found that the rate of cartilage repair and repair after injury was between 7 and 14 days, with a maximum recovery time of 14 days. We have selected 14 days for our research and then evaluated and expressed the gene.(24)

AdilaA Hamid et. al(2012) to examine the ability of human adipose-derived stem cells to characterize and express chondrogenic genes during cartilage differentiation, human adipose-derived stem cells were successfully differentiated into cartilage and bone lineages. Human fat-derived stem cells accumulate after the induction of cartilage and form a dense matrix. The expression of chondrogenic genes (type II collagen, Aggrecan nucleus protein, type XI collagen, COMP was significantly higher. However, a significantly increased expression of type X collagen-induced chondrogenic induction was observed. The experiments themselves showed that the expression of cartilage genes increased compared to the control group.(25)

Hanxiang Le et. al(2020) To study mesenchymal stem cells for cartilage regeneration, they stated that fibrin was usually the cell production system during the cartilage repair process to produce new cartilage matrices. In addition, fibrin replicates MSC, which is in line with our work in that the scaffold used by Mahm was selected based on these characteristics.(26)

JihangDai et. al(2021) study on licorice and Glabridin, which are effective compounds in licorice, and found that glabridin expressed the expression level of genes related to extracellular matrix (ECM), collagen II, aggregate (ACAN), SRY. Increased box 9 (SOX9) and proteoglycan 4 (PRG4).(27)

Maryam Kazemi et. al(2020) study on wound healing by an emulsion of a combination of lavender and licorice stated that real-time PCR data showed that nanoemulsion and technical groups Twins have shown the best results in increasing collagen gene expression compared to other groups. Grain tissue formation was also faster than collagen in the nanoemulsion and phenytoin groups. It can be said that based on the results of our test, licorice also caused the expression of collagen genes(28)

Ren-Hao Jiang et. al(2020) investigate the effects of glycyrrhizin on the glands of human OA cells reported that glycyrrhizin, a compound extracted from licorice, has various important biological activities.

It has antioxidant properties and anti-inflammatory functions. The study found that glycyrrhizin reverses the degradation of aggregates and collagen II and improves their production. In summary, all results suggest that glycyrrhizin may be a potential treatment for OA. . According to our real-time test data, which shows the expression of collagen and if genes more than the control group, we can say that licorice can be an effective factor in the expression of cartilage genes.(29)

Conclusion

Finally, we can conclude that the simultaneous use of both extracts improves the expression of cartilage-specific genes and, to a very small extent, promotes cells to hypertrophy, which we can also improve by changing the doses used. The viability of cells during concomitant use of the extract was higher than other groups, which can be concluded that the use of the extract with fibrin scaffold was effective on cell survival.

Declarations

Funding:

This work was supported by Shahid Sadoughi University of Medical Sciences, Yazd, Iran

Abbreviations

CollX(collagenx).coll2(collagen2). Licorice (Lic L) Elaeagnus Angustifolia fruit(of E. angustifolia L)

Availability of data and materials

All data included in this study are available from the corresponding author by reasonable request.

Ethics approval and consent to participate

Ethics code 7880 received from the Research Council of Shahid Sadoughi University of Medical Sciences, Yazd

Competing interests

The authors declare that they have no competing interests.

Consent for publication

All authors are fully satisfied with the release of the version

Authors' contributions

AR. MP - MY : conceptualization, experimental set-up, data interpretation, manuscript writing, and figure preparation. AR: experimental work, data analysis and interpretation. AA,AM.MM,KR : data analysis,

interpretation, manuscript and figure formatting. All authors reviewed and approved the final version of the manuscript

Acknowledgements

The authors also thank all laboratory colleagues for their assistance. We also express our appreciation to dr.A Hsanpour and F khosravi for their valuable assistance

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Tables

Table 1-The final volume of each reaction in Real Time PCR

Component	Volume	Component	Volume
SYBR Green	10	cDNA	1.5
Primer F	0.5	Primer R	0.5
DEPS	7.5	Total reaction	20

Table 2-Sequence of primers

Gene	Primer Sequences (Forward)	Gene	Primer Sequences (Reverse)
collagen II-F	CTGGTGATGATGGTGAAG	collagen II –R	CCTGGATAACCTCTGTGA
collagen x –F	AGAATCCATCTGAGAATATGC	collagen x – R	CCTCTTACTGCTATACCTTTAC
sox-9 –F	TTCAGCAGCCAATAAGTG	sox-9 –R	TTCAGCAGCCAATAAGTG
Aggrecan-F	GTGGGACTGAAGTTCTTG	Aggrecan-R	GTTGTCATGGTCTGAAGTT
GAPDH-F	AAGCTCATTTCTGGTATG	GAPDH-R	CTTCCTCTTGTGCTCTTG

Figures

Figure 1

The method of separating the stem cells of fat and tissue passage

1) Cut the fat into small pieces. 2) Add 0.5 mg of collagenase 2 per gram of fat. 3) Place in the incubator for 60 to 90 minutes. 4) Add the same amount of FBS. 5) for 15 minutes at 2200 RPM in a centrifuge. 6) Hold the third phase and remove the rest of the contents of the Falcon tube. 7) Add 6 ml of culture medium. 8) Transfer the cells to the flask. 9) Place in the incubator.

Figure 2

Fibrin scaffolding and differentiation of fat cells towards cartilage

Figure 3

Comparison cell viability of Trypan blue Results Between Groups

Figure 4

Quantitative Expression Status of the Agg, Sox 9, Coll2, Coll10 Genes Analyzed by Real-Time PCR

***=P-value =0.001		*=P-value =0.05	
a = Significance of the control group compared to other groups		d = Significance of licorice group compared to other groups.	
b = Significance of the composition group compared to other groups		c = Significance of Eleagnus angustifolia L compared to other groups.

Figure 5

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