

# Human Umbilical Cord Mesenchymal Stem Cells-Derived Small Extracellular Vesicles Can Be Considered as Cell-Free Therapeutics for Angiogenesis Promotion

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## Abstract

**Objective:** Angiogenesis has critical roles in several physiological processes. Restoring angiogenesis in some pathological conditions such as a few vascular diseases can be a therapeutic approach to controlling this issue. Mesenchymal stem cells (MSCs) secrete specific intracellular products known as extracellular vesicles (EVs) with high therapeutic potential which compared to their source cells, do not have the limitations of cell therapy. The angiogenic effect of the human umbilical cord MSCs (hUCMSCs)-derived small EVs are evaluated in the present work. Aim of this research is to show that hUCMSCs-derived small EVs cause differentiation of genes involved in angiogenesis like *FGFR-1*, *FGF*, *VEGF*, and *VEGFR-2*.

**Materials and Methods:** In this experimental study, MSCs were isolated from the human umbilical cord, and after confirming their identities, their secreted EVs (including exosomes) were extracted by ultracentrifugation. The isolated small EVs were characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM), bicinchoninic acid assay (BCA), and Western Blotting. Then, the human umbilical vein endothelial cells (HUVECs) were treated with derived small EVs for 72 hours, and the expression of the angiogenic factors including *FGFR-1*, *FGF*, *VEGF*, and *VEGFR-2* was evaluated by quantitative real-time-polymerase chain reaction (qPCR). Angiogenesis was also evaluated via a tube formation assay.

**Results:** The results demonstrated that *FGFR-1*, *FGF*, *VEGF*, and *VEGFR-2* could be elevated 2, 2, 3.5, and 2 times, respectively, in EVs treated HUVECs, and derivative EVs can encourage tube formation in HUVECs.

**Conclusion:** These findings imply that hUCMSCs-derived small EVs are valuable resources in promoting angiogenesis and are very promising in cell-free therapy.

**Keywords:** Angiogenesis, Exosome, Extracellular Vesicles, hUCMSCs, Vascular Endothelial Growth Factor

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## Introduction

The process of angiogenesis encompasses new blood vessel construction from old veins (1). Angiogenesis is a complex multi-stage process in which inactive endothelial cells are stimulated with angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF or bFGF), hepatocyte growth factor (HGF), transforming growth factor-beta (TGF- $\beta$ ), and are organized in a tubular structure (2). Among angiogenic inducing factors, VEGF and FGF are very important and well-known factors. As a crucial physiological process, angiogenesis plays a significant role in rehabilitating tissues, healing wounds, menstrual cycles, and helping in the treatment of some diseases such as cancer. Increasing angiogenesis is a therapeutic approach for people who suffer from acute ischemia (3). Angiogenesis is dependent on the exact balance between stimulation and natural inhibitors in the body. If the balance deviates from its normal state, some pathological conditions such as atherosclerosis, proliferation, and metastasis of tumors occur. On the other hand, inducing angiogenesis can be supportive in some diseases. Therefore, it is necessary to

study the mechanisms of angiogenesis and its improvement in some pathological conditions (1, 4).

Cell therapy has been considered for various kinds of diseases during the last decades, and for this purpose, different stem cells (especially mesenchymal stem cells, MSCs) were used as a promising therapeutic approach in several studies (5).

MSCs are multipotent adult non-hematopoietic stem cells (HSC) with high differentiation, proliferation, and self-renewal. These cells can be isolated from tissues with mesodermal origins, including liver, spleen, adipose tissue, thymus, umbilical cord blood, Wharton's jelly, placenta, lung, peripheral blood, dental pulp, and other tissues (6, 7). The three most important and easiest accessible sources are bone marrow, adipose tissue, and umbilical cord. Umbilical cord blood and Wharton's jelly are the most abundant sources for MSCs. MSCs from different sources are recognized with the same phenotype (spindle appearance; similar to fibroblasts) but with different functions; for example, umbilical cord MSCs have been shown to induce angiogenesis (8, 9). MSCs

are important stromal cells in the bone marrow that can feed and revitalize bone marrow (7). They have an important role in the regeneration of tissues such as bone, cartilage, fat, muscle, and tendon. These cells are also capable of differentiating into adipocytes, osteoblasts, and chondrocytes *in vitro*. Furthermore, they are capable of migrating to damaged tissues and releasing cytokines, inflammatory mediators, extracellular matrix compounds, and angiogenic and anti-fibrotic factors (6). These cells lack the major histocompatibility complex-II (MHC-II) and stimulus-assisting molecules like CD86, CD40, and CD80 (10). These features made them a good candidate for the treatment of some diseases, as they were cured by MSCs. Although there are some drawbacks to cell therapy, including safety issues, limitations of cell transplants, and graft versus host disease (GVHD) risk (11).

MSCs exert a unique function by secreting various cytokines, growth factors, microRNAs, and extracellular vesicles (EVs) (12). The EVs are spherical lipid bilayer vesicles containing biogenic substances like proteins, mRNA, and microRNA. Their average size differs from 30 nanometers to several micrometers. EVs are categorized according to their size and cell source and are comprised of exosomes, microvesicles or microparticles, apoptotic vesicles, etc (13). Exosomes, for example, belong to small EVs with a diameter of about 30-100 nanometers. They are derived from endosomes and buds from the plasma membrane with the same antigens as their cells of origin's membrane (14). Common markers between exosomes are CD9, CD81, and CD63 (tetraspanins) (15). It was shown that EVs have different functions, such as removing unwanted substances from the cells. Exosomes derived from MSCs perform similarly to MSCs, such as tissue injury regeneration, induction of angiogenesis and neurogenesis, and immune modulation. Given the specific disadvantages of cell therapies, EVs (especially exosomes), as cell products similar to their source cells, have been gaining attention as a safe alternative therapeutic approach to the paracrine function of MSCs (16).

In general, small EVs are diagnostic and prognostic biomarkers and an effective means for transmitting therapeutic agents. They can be used to diagnose and treat various inflammation, heart and metabolic diseases, neurodegenerative disorders, and malignant neoplasms (17).

With the available evidence on the benefits of using MSCs to enhance angiogenesis, we have decided to assess the effect of hUCMSCs-derived small EVs in the induction of angiogenesis *in vitro*.

## Materials and Methods

### Isolation and culture of mesenchymal stem cells from human umbilical cord

In this experimental study, The UC sample was obtained

with the personal consent of donors in Mahdijeh hospital (Tehran, Iran) after the child's birth. The applied protocols were accepted by the Ethics Committee of Tarbiat Modares University (IR.MODARES.REC.1397.237). Under sterile conditions, the umbilical cord specimen was chopped on a plate using a bistoury, and one-milliliter collagenase type II (1 mg/mL, Sigma, USA) was added and incubated for one hour at 37°C and 5% CO<sub>2</sub> until it was dissolved; it was vortexed every 15 minutes and then centrifuged at 300 g for 5 minutes. The cells (pellet) were cultured in a 75 cm<sup>2</sup> (T75) culture flask. Cell culture medium included DMEM/F12 (Bio-Idea, Iran) supplemented with 10% fetal bovine serum (FBS) (Gibco's) and 100 µl of penicillin/streptomycin (Sigma, USA). After three passages, the cells were used for further tests (18).

### Identification of mesenchymal stem cells

MSCs were identified by assessing the expression of CD31, HLA-DR, CD34, CD105, CD90, and CD73 markers with the use of flow cytometry after tagging by a fluorescent antibody (BioLegend). Briefly, cells were counted after trypsinization and suspended in 3% bovine serum albumin (1×10<sup>5</sup> cells per 100 µl). Then the desired antibodies and their control isotype were added to 100 µl of the prepared suspensions and after refrigeration for 45 minutes, they were washed with PBS, and then flow cytometry analysis was performed on the samples.

### The differentiation of mesenchymal stem cells to osteoblasts and adipocytes

The differentiation of cells into adipocytes and osteoblasts was done by adding the differentiation media and then the evaluation was done by staining with Oil-Red-O 5% and Alizarin-Red S 2% (Sigma Aldrich, USA) (2, 19). Oil-Red-O and Alizarin-Red are differential dyes that redden the color of cells when they bind to fat droplets in adipocytes or calcium sediments in osteoblasts, respectively.

For cell differentiation into osteoblasts, a medium composed of DMEM supplemented with 10% FBS, ascorbate-2-phosphate factors (0.2 mM/ml, Sigma Aldrich, USA), dexamethasone (0.1 mM, Sigma Aldrich, USA), and 10 mM beta-glycerol phosphate (Sigma Aldrich, USA) were used.

The medium for differentiating cells into adipocytes included DMEM, FBS (10%), insulin (1 µg/ml) (Sigma Aldrich, USA), dexamethasone (7-10 M, Sigma Aldrich, USA), and indomethacin (2 µM, Sigma Aldrich, USA).

The MSCs were cultured in 6 well plates after the confluency of 70%, the medium was removed entirely, and 2 ml of osteoblastic differentiation medium or adipocytic differentiation medium were added. The medium was changed twice a week for three weeks. Then staining was performed on the 21<sup>st</sup>-day.

### Isolation of small extracellular vesicles

With the current methods, exosomes can not be isolated from other kinds of EVs; for example, by ultracentrifugation, small EVs can be obtained instead of pure exosomes.

The third passage of cells was used for small EVs isolation; after achieving 40% cell confluency, the cell culture medium was changed with DMEM/F12 containing 10% exosome-free FBS (prepared by ultracentrifugation of FBS at 120,000 g for 2 hours and discarding the sediment and filtering the supernatant), and then the medium was collected when cells reached 80% density. In order to isolate small EVs, a standard method was used by ultracentrifugation.

Centrifugation was performed at 300g for 10 minutes in the first stage, and the second stage was performed at 10000g for 20 minutes to separate cells and debris. The supernatant was then filtered (0.22 Millipore filter) to separate large EVs. Then, two ultracentrifugation steps were performed at 100000g for 70 minutes at 4°C (Beckman 60 TI) to pellet the small EVs. At each stage, the sediments containing small EVs were washed with 1 ml phosphate-buffered saline (PBS, pH=7) (20).

### A quantitative and qualitative investigation of isolated extracellular vesicles protein concentration assay

The total protein concentration was determined by the Bicinchoninic Acid Assay (BCA) method. The BCA Protein Assay Kit (Biobasic, Canada) was utilized, and tests were done according to the manufacturer's protocol. The standard curve was drawn using successive dilutions of BSA protein with a certain concentration. The samples were measured by ELISA reader (BioTeK) at 562 nm and compared with the standard curve.

### Extracellular vesicles' size determination

The isolated EVs were diluted in 1 ml of PBS (pH=7) and size was examined using dynamic light scattering (DLS, Zetasizer, Malvern).

### Extracellular vesicles' morphology evaluation

The morphology and structural integrity of EVs were visualized by transmission electron microscopy (TEM, Leo 906).

### The immunodetection of extracellular vesicles by western blotting

Assessing specific markers on isolated EVs was done by western blotting.

After the separation of cellular proteins, the intended proteins can be detected with the help of specific antibodies. For the identification of EVs, tetraspanin proteins, CD9, CD63, and CD81, are the most common proteins that can be visualized by Western blotting (21).

The expression of CD63 and CD9 was evaluated in this study, as follows: EVs were lysed by lysis buffer supplemented with proteinase inhibitors to obtain the EVs' total proteins; the isolated proteins were resolved over sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Then in the blotting stage, the resolved proteins were transferred to the polyvinylidene difluoride (PVDF) membrane and treated with primary antibodies (anti-CD9 and anti-CD63 antibody) (1:1000; Santa Cruz Biotechnology, CA), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (SinaClon, Tehran, Iran). The luminescence tagged proteins were visualized using the ECL system. Beta-actin was served as the protein of control.

### Treatment of HUVEC cells with extracellular vesicles

HUVECs can be used to study the function and pathology of endothelial cells, such as angiogenesis. For evaluating the angiogenic effects of EVs, HUVECs (purchased from a stem cell research center, Tehran, Iran) were treated with EVs, and then the angiogenesis-related genes expression and angiogenesis were examined by quantitative real-time-polymerase chain reaction (qPCR) and tube formation assay, respectively.

### Investigation of the angiogenesis-related genes expression in with extracellular vesicles-treated HUVECs

About 100000 cells of fresh HUVECs were transferred to a 24-well plate and 400 µl EVs solution (equal to 50 µg/ml EVs) was added to each well and incubated for 72 hours, which is the suitable time for the entrance of EVs into the cells and gene expression. The untreated cell wells were considered as the negative control.

Changes in the expression of angiogenic genes, *VEGF*, *FGF*, *FGFR-1*, and *VEGFR-2*, were measured by real-time PCR, as follows: RNA extraction was performed by TRIzol solution (Yekta Tajhiz Azma, Iran) based on the manufacturer protocol. Next, cDNA was synthesized (Pars Tous cDNA synthesis kit, Iran). Then the cDNAs' quality was assessed by PCR, and finally, the target genes were quantified by real-time PCR using the SYBR Green master mix (Yekta tajhiz azma, Iran) and Step One Real-time PCR system (Applied Biosystems, USA). The real-time PCR results were analyzed using the  $2^{-\Delta\Delta CT}$  method and expressed as relative expression of threshold cycle (CT) value (treated groups compared to control group). Control genes (or housekeeping genes) are frequently used to normalize mRNA levels between different samples. However, the expression level of these genes may vary among tissues or cells. The cells we used were HUVECs for which the most common housekeeping gene is hypoxanthine guanine phosphoribosyl

transferase (*HPRT1*) (22). The primers used to perform the real-time PCR are summarized in Table 1.

**Table 1:** The primers used for real-time polymerase chain reaction

Gene name	Primer sequences (5'-3')	T <sub>m</sub> (°C)
<i>VEGFA</i>	F: GCCAGCACATAGGAGAGATGAGC R: CGGCTTGTCACATTTTTCTGGT	60
<i>VEGFR-2</i>	F: ACTCTTCAAATTACTTGCAGGGG R: CTCCAGTGTCAATTCGATCAC	60
<i>bFGF</i>	F: GGCTTCTCCTGCGCATCCA R: GCTCTTAGCAGACATTGGAAGA	60
<i>HPRT1</i>	F: CCTGGCGTCGTGATTAGTG R: TCAGTCCTGTCCATAATTAGTCC	60
<i>FGFR-1</i>	F: AAACCGTATGCCGTAGC R: TCCATTATGATGCTCCAGGTG	61

### Tube formation

The angiogenic potential of EVs was assessed by a tube formation assay to see if they can form a capillary network. HUVECs, as a source of endothelial cells, were cultured in Matrigel (BD Biosciences) coated 24-well plates, and 50 µg/ml of EVs were added to sample wells; the controls remained untreated. The cell culture medium contained high-glucose DMEM supplemented with 10% FBS. Cell morphology changes were seen under an inverted microscope for 24 hours.

### Statistical analysis

All the treated and control samples were tested in triplicate. The real-time PCR results were analyzed using the  $2^{-\Delta\Delta CT}$  method and expressed as relative expression of treated groups compared to the control group. The evaluated data were shown as mean ± SD. Data were analyzed by the ANOVA statistical method using GraphPad Prism 8 software (GraphPad Software Inc, CA, USA), and a P<0.05 was considered statistically significant.

## Results

### Identification of umbilical cord-isolated mesenchymal stem cells

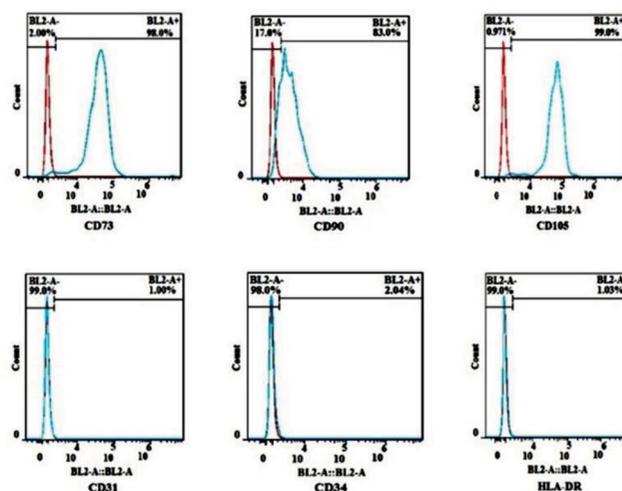
#### Evaluation of cellular immunophenotype

The data from the flow cytometry examination of human UC-derived cells are shown in Figure 1A. The tested cells were negative for HSC markers, CD342.04%) ), CD31 (1.00%), and HLA-DR (1.03%) and positive for MSC markers, CD90 (83%), CD73 (98%), and CD105 (99.0%), confirming the identification of MSCs.

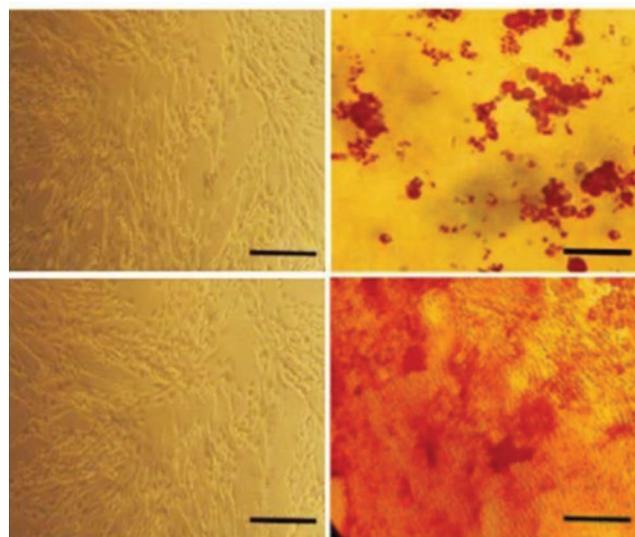
### Investigation of differentiation properties

Differentiation of umbilical cord MSCs (UCMSCs) under specific differentiation media into adipocyte and osteoblast cells was assessed after 21 days using diagnostic dyes, Oil-red O and Alizarin-Red, respectively. As seen in Figure 1B, the isolated cells could be differentiated to either osteoblast or adipocyte cells in contact with the specific differentiation medium, which is characteristic of MSCs.

#### A



#### B



**Fig.1:** Identification of isolated cells from the human umbilical cord. **A.** Flow cytometry histograms for evaluating HSC markers, CD34, CD31, HLA-DR, and MSCs surface markers, CD73, CD90, and CD105. The cells were negative for hematopoietic and positive for MSCs markers. **B.** In the proximity of differentiation media (after 21 days), isolated UCMSCs could differentiate into adipocyte or osteoblast cells, shown in upper and lower panels, respectively. The left panels in each group are the control cells (magnification, 200X, scale bar: 50 µm). Alizarin-Red and Oil-red O were used to stain calcium-containing osteoblast and adipocytes, respectively. All the experiments were tested in triplicate. HSC; Hematopoietic stem cell, MSCs; Mesenchymal stem cells, and UCMSCs; Umbilical cord mesenchymal stem cells.

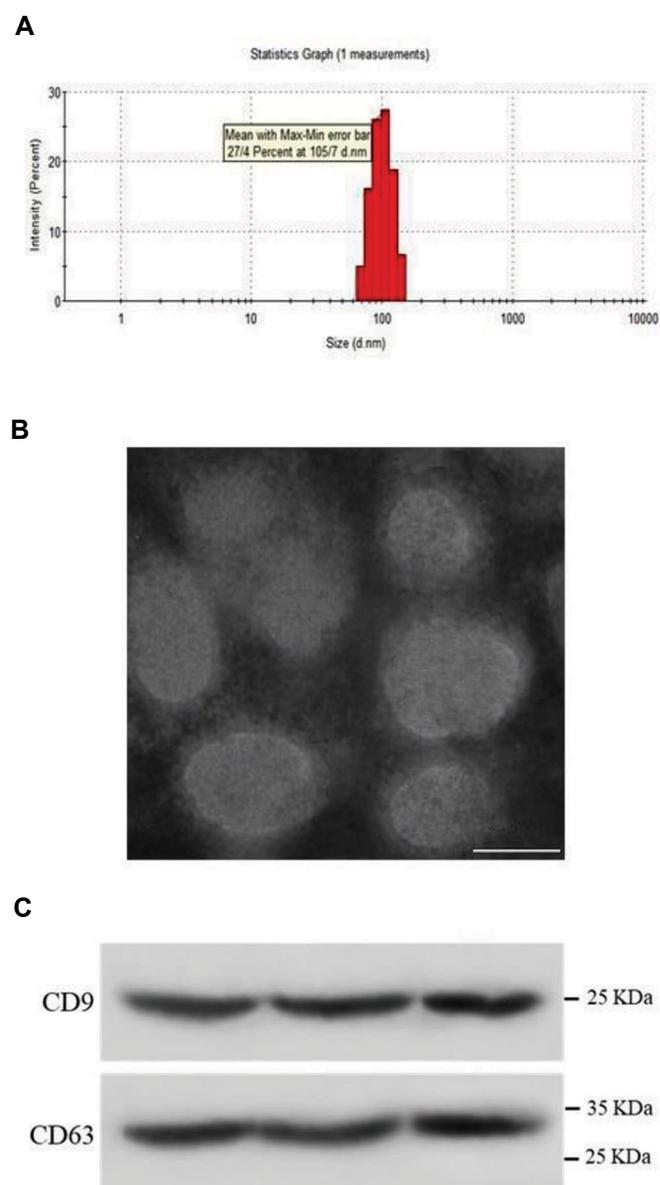
## Characterizing the extracted extracellular vesicles

### Determination of the diameter of extracellular vesicles

As shown in Figure 2A, most of the EVs populations have an average diameter of 105.7 nm. DLS Technique was used to obtain the data.

### Verification of the quality and structure of the extracellular vesicles

According to the results obtained through the TEM technique, Figure 2B shows that EVs have spherical structures, and the membrane is preserved without being damaged.



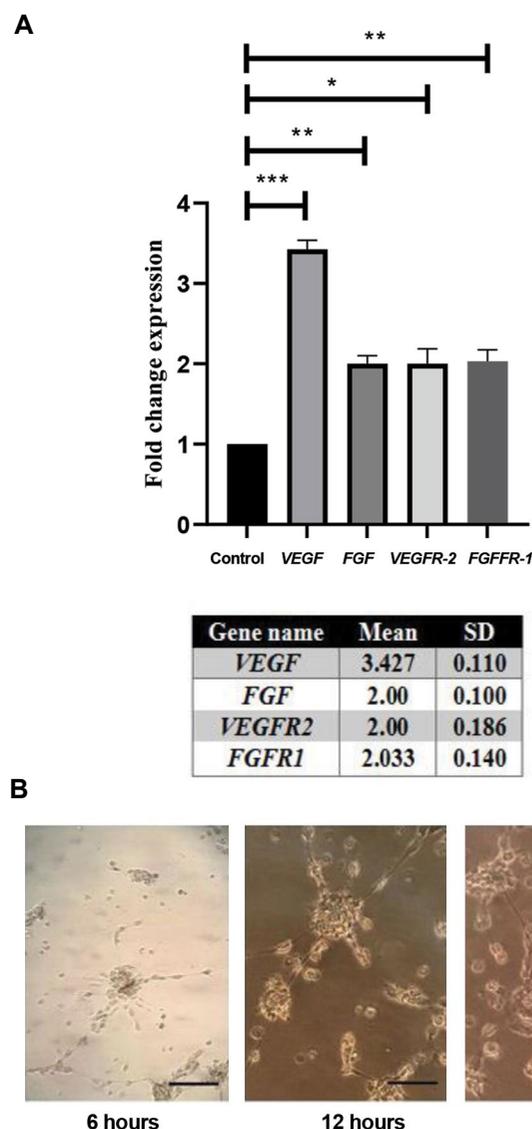
**Fig.2:** Characterization of the hUCMSC-derived EVs. **A.** Diameter of isolated EVs was measured by DLS; as shown, the majority of the EVs population has an average diameter of 105.7 nm. **B.** According to the TEM pictures the EVs have a spherical structure, and the membrane is preserved without being damaged (scale bar: 100 nm). **C.** Identification of exosomal markers, CD9 and CD63, by Western blot analysis. All the experiments were tested in triplicate. hUCMSC; Human Umbilical cord mesenchymal stem cell, EVs; Extracellular vesicles, DLS; Dynamic light scattering, and TEM; Transmission electron microscopy.

### Determination of the total protein concentration of extracellular vesicles

The BCA method measured a concentration of 203.33  $\mu\text{g/ml}$  of EVs, comparing their optical absorption with the standard curve.

### Western blotting

Western blot results (Fig.2C) showed that CD9 and CD63 (tetraspanins) were highly expressed on the surface of EVs indicating the presence of exosomes.



**Fig.3:** Evaluation of the angiogenic effect of the hUCMSC-derived EVs. **A.** Detection of *VEGF*, *FGF*, *FGFR-1*, and *VEGFR-2* genes expression changes compared to the control group showed that the expression of these genes in EVs-treated HUVEC cells increased significantly. Data are presented as the mean  $\pm$  SD and each group was compared with the control group; the meaningful changes are represented by asterisks (\*;  $P < 0.05$ , \*\*;  $P < 0.01$ , \*\*\*;  $P < 0.001$ ) and the exact numbers of P value were 0.0007 for *VEGF*, 0.0033 for *FGF*, 0.0114 for *VEGFR2*, and 0.0061 for *FGFR1*. **B.** Sequential tube formation after HUVECs treatment with EVs. Cell elongation, as well as connection to form a loop is apparent in the figure. Morphological changes in the cells were observed under the inverted microscope for 24 hours (scale bar: 50  $\mu\text{m}$ , magnification, 100x). All the experiments were tested in triplicate. hUCMSC; Human Umbilical cord mesenchymal stem cell and EVs; Extracellular vesicles.

## Angiogenic effect of extracellular vesicles

### RNA extraction and Real-time polymerase chain reaction

The expression of some critical angiogenic markers was measured in HUVECs after treating them with EVs. The qRT-PCR results were reported as relative gene expression (using the  $2^{-\Delta\Delta Ct}$  analysis method) and normalization by the housekeeping gene *HPRT1*. Data revealed that expression of *FGFR-1*, *FGF*, *VEGF*, and *VEGFR-2* genes were increased 2, 2, 3.5, and 2 fold, respectively, in treated cells compared to control groups (Fig.3A). According to these data, VEGF expression has increased more than the other genes.

### Tube formation assay

Figure 3B demonstrates the tube formation test results. Increased cell branches, elongation, and cell connections in the form of a loop are evident, confirming tube formation.

## Discussion

Angiogenesis is a vital event in the body's physiological processes during life, such as wound healing and tissue regeneration. Besides, increasing angiogenesis is highly effective in controlling many diseases, such as acute ischemia. Given the importance of angiogenesis, it can be considered a new therapeutic approach for some pathological conditions.

Although many factors increase angiogenesis, VEGF and FGF are the most critical and well-known factors (3, 23, 24).

MSCs are important as feeders of HSCs and play an important role in the regeneration of tissues by secretion of growth factors, EVs and cellular contact (7). Due to their ability to differentiate, autologous transplantation, and their unique properties, MSCs have received attention in the treatment of some diseases (25), such as bone tissue disorders (26), immune disorders (27) and cardiovascular diseases (28). Three important sources of MSCs are fat, bone marrow, and umbilical cord (7). MSCs of different sources have been shown to increase angiogenesis. Alvaro-Afonso et al. (29) examined the effect of adipose-derived MSCs (AMSCs) on diabetic foot ulcers (DFUs). Based on this study, it was concluded that AMSCs are promising for the treatment of DFUs. In another study by Zhang et al. (30), the effect of MSCs on the treatment of cardiovascular and ischemic diseases was studied. MSCs could differentiate to smooth muscle cells (SMCs) and endothelial cells (ECs) and release some factors which help cardiovascular regeneration.

A comparison of bone marrow-derived MSCs with umbilical cord MSCs by Wang et al. (31) showed that UCMSC could also induce angiogenesis and improve blood flow. It was demonstrated that UCMSCs differentiate into the three germ layers and enhance tissue repair and modulate immune responses. These cells showed a more substantial angiogenic effect than BMSCs. In these

articles, different applications of MSCs with different sources were shown; these cells have been used to stimulate angiogenesis and treat various diseases such as diabetic foot ulcers. In fact, the purpose of this study was to compare the sources of MSCs used in previous articles with the umbilical cord. The accessibility, ease of use, and comfortable extraction of MSCs make them a more suitable source as compared to previously mentioned sources for induction of angiogenesis. Other applications of MSCs are also discussed in these articles.

Considering the disadvantages of cell therapy, the idea of using a type of EVs, like exosomes, was taken into account as a new therapeutic strategy. The role of MSCs-derived exosomes was first studied in 2010 in the mouse model of myocardial ischemia/reperfusion damage, and then it was assessed in other disease models (32). EVs have different functions, such as intercellular signaling, and have unique properties in terms of concentration, appearance, and markers (13, 14). EVs also have other advantages over cell therapy, such as low immunogenicity (without MHC I, II) (33), low toxicity, facilitating the uptake of RNA and protein into damaged cells, the ability to carry large cargo, and strong protection from enzyme degradation, inherent stability and the ability to cross the blood-brain barrier (34).

Exosomes as small EVs have the same therapeutic effects on diseases as their source cells (16). There have been numerous studies on MSC-derived exosomes, which used various sources of MSCs for angiogenesis induction, and their benefits have been identified. The effect of UCMSCs-derived exosomes on acute myocardial infarction has been investigated. According to the results of such experiments, hUCMSC exosomes could protect systolic cardiac function and reduce cardiac fibrosis by protecting myocardial cells against apoptosis, which might be related to regulating Bcl-2 family gene expression and promoting angiogenesis (35). In 2016, Liang et al. (36) examined the effect of exosomes derived from adipose MSCs (adMSCs) on promoting endothelial cell angiogenesis. The researchers discovered that adMSC-derived exosomes (adMSCs-Exo) could be absorbed by endothelial cells and significantly enhance *in vitro* and *in vivo* angiogenesis. The study has also shown that adMSCs-Exo can translocate miR-125a to endothelial cells and induce angiogenesis by suppressing angiogenic inhibitor delta-like 4 (DLL4). Huang et al. (37) examined the angiogenesis and neurogenesis properties of bone marrow-derived MSCs exosomes modified with miR-126 in spinal cord injury (SCI) rats. These exosomes were used as a means of microRNA transfer for the first time. It was found that after spinal cord injury, MSC-derived exosomes (MSC-Exo) could transfer miR-126 to the affected spinal cord in rats, and exosomes containing miR-126 reduce the size of the lesion and improve function after SCI.

In this study, we used the umbilical cord (Wharton's jelly) because angiogenesis is abundant in this tissue and its other features are being easily accessible, non-invasive

harvesting, better viability, and consistent therapeutic properties (38, 39). We therefore used hUCMSCs-derived small EVs. The isolated EVs were identified. They had desirable properties, including a mean diameter of about 105 nm, the appearance of the bilayer membrane, and the presence of CD9 and CD63 markers. They were able to induce angiogenesis in HUVECs, as proved by increased expression of angiogenic genes, *FGFR-1*, *FGF*, *VEGF*, and *VEGFR-2*, and tube forming colonies. Angiogenesis is mediated mainly by VEGFA/VEGFR2 signaling. VEGFA causes endothelial proliferation, migration, and survival via activation of VEGFR2 and its downstream signal transduction pathways. VEGFA is critical for physiological and pathological processes. So VEGFA expression has been increased more (40). Analysis of tube formation images confirmed that hUCMSCs-derived EVs could induce tube branching from colonies of HUVECs.

In general, angiogenesis is a complex multi-stage process involving the activation of inactive endothelial cells, proliferation, migration, germination of endothelial cells, formation of tubular structures, maturation, and stability of newly formed buds, respectively. In this study, the signaling pathways are such that angiogenic factors are secreted by hUCMSCs-derived EVs. The secretion of these factors activates their receptors on endothelial cells (HUVEC cells) and thus begins angiogenesis. As a result, the increased expression of the desired genes compared to the control group was investigated and proven in our study. Thus, this study provided a new and useful application of hUCMSCs-derived EVs, i.e., induction of angiogenesis, which can be valuable in cell-free therapy. However, factors increasing angiogenesis are not studied in this study and need further investigation; besides, animal studies must be done to examine the potential of tumorigenesis of UCMSCs-derived exosomes.

## Conclusion

According to the present study, it has been demonstrated that hUCMSCs-derived small EVs could lead to increased expression of angiogenesis-related genes and the angiogenesis process. Based on these results and studies at the molecular level, small EVs derived from the hUCMSCs can be a good candidate for angiogenesis induction which has valuable potential as a novel cell-free medication.

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## Authors' Contributions

M.S.Z., S.A.; Contributed to the conception and design. M.S.Z.; Was responsible for overall supervision. S.D.; Performed the experimental work. S.D., S.A., N.T.; Participated in data and statistical analysis, and interpretation of data. N.T.; Drafted the manuscript. All

authors read and approved the final manuscript.

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