ROMM
Revista de Osteoporosis y Metabolismo Mineral

Original

Circulating extracellular vesicles affect mesenchymal stromal cell differentiation and angiogenesis. Potential use in bone regeneration

Sara Oliva-Lozano[1], Victoria Pulido-Escribano[1], Bárbara Torrecillas-Baena[1], Alfonso Calañas-Continente[1,2], Feliciano Priego-Capote[3,4], Marta Camacho-Cardenosa[1], Antonio Casado-Díaz[1,2,4]

[1]Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC). Córdoba, Spain. [2]Unit of Clinical Management of Endocrinology and Nutrition. Hospital Universitario Reina Sofía. Córdoba, Spain. [3]Department of Analytics Chemistry. Rabanales Campus. Universidad de Córdoba. Córdoba, Spain. [4]Centro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable (CIBERFES). Córdoba, Spain

Abstract

Introduction: the use of extracellular vesicles (EVs) has a high potential in regenerative medicine. Although mainly those derived from mesenchymal stromal cells (MSC) have been studied, circulating EVs from umbilical cord blood (UCBEV) or from healthy young adults (peEV) also contain factors that can favor tissue regeneration. This study evaluates the effect of UCBEV and peEV on MSC differentiation to osteoblasts and adipocytes, and endothelial cell angiogenesis.

Material and methods: MSC cultures were treated with UCBEV and peEV during differentiation into osteoblasts or adipocytes. The expression of osteoblastic or adipogenic genes was studied. Mineralization and lipid droplet formation were quantified. Umbilical cord vein endothelial cells (HUVEC) were evaluated in angiogenesis assays.

Results: UCBEV and peEV did not affect MSC viability, but peEV increased HUVEC viability. In osteoblasts, collagen type I alpha 1 (COL1A1) expression was increased by peEV, but mineralization was not affected. In adipocytes, adipose triglyceride lipase (ATGL) and fatty acid-binding protein 4 (FABP4) expression was inhibited, and lipid droplet formation was decreased with both types of EV. In HUVEC, UCBEV and peEV induced angiogenesis.

Conclusion: the results suggest that both types of EVs, from abundant sources, without major ethical issues and easy to isolate, have high potential in regenerative medicine applied to bone, inhibiting bone marrow adiposity and favoring angiogenesis.

Keywords:
Mesenchymal stromal cells. Adipocytes. Osteoblasts. Circulating extracellular vesicles. Exosomes. Angiogenesis.

Received: 11/01/2024 • Accepted: 02/09/2025

Sara Oliva-Lozano and Victoria Pulido-Escribano contributed equally to this study.

Marta Camacho-Cardenosa and Antonio Casado-Díaz are the corresponding authors of this article.

Funding: This research has been funded by CIBER "Frailty and Healthy Aging" (CIBERFES, CB16/10/00501) and the Carlos III Health Institute (ISCIII, PI21/01935), Ministry of Economy and Competitiveness (MINECO), Spain, and the European Union (EU).

Ethical aspects: This study has been conducted in compliance with the Declaration of Helsinki. All participants provided written informed consent prior to their inclusion in the study.

Acknowledgments: FEIOMM Translational Research Scholarship 2022.

Conflict of interest: The authors declare no conflict of interest.

Artificial intelligence: The authors declare not to have used artificial intelligence (AI) or any AI-assisted technologies in the elaboration of the article.

Oliva-Lozano S, Pulido-Escribano V, Torrecillas-Baena B, Calañas-Continente A, Priego-Capote F, Camacho-Cardenosa M, Casado-Díaz A. Circulating extracellular vesicles affect mesenchymal stromal cell differentiation and angiogenesis. Potential use in bone regeneration. Rev Osteoporos Metab Miner 2025;17(1):19-30

DOI: 10.20960/RevOsteoporosMetabMiner.00066

Correspondence:
Antonio Casado-Díaz. Unit of Clinical Management of Endocrinology and Nutrition. Hospital Universitario Reina Sofía. Edificio IMIBIC. Menéndez Pidal, s/n. 14004 Córdoba, Spain
e-mail: bb1cadia@uco.es

INTRODUCTION

The bone system depends on a balance between bone formation and resorption. When this balance is disrupted by trauma in the form of bone fractures, this tissue has the ability to self-renew (1). In this healing process, various cell types, extracellular matrices, and signaling molecules are involved (2), across the three main phases that occur: the inflammatory phase, the reparative phase, and the remodeling phase. In the reparative phase, osteoprogenitor cells and undifferentiated mesenchymal cells are induced to differentiate into osteoblasts, rather than into other cell types such as adipocytes (3). Furthermore, the formation of new blood vessels, which supply oxygen and nutrients necessary for bone formation, is crucial in the regenerative process (2). However, in some cases, healing delays, non-union fractures, or bone diseases (osteoporosis, osteonecrosis, or cancer) can occur. These conditions present significant morbidity and substantially reduce the activity and quality of life of patients suffering from them. Therefore, new therapeutic strategies are needed to reduce prolonged immobilization or repeated surgical interventions. These could also pose a significant cost to healthcare systems and society (4).

Recent advances in tissue engineering have proposed the combination of different cell types with synthetic biomaterials as an alternative to bone grafts. Specifically, mesenchymal stem cells (MSC) derived from bone marrow have been proposed for the treatment of various pathologies due to their differentiation, regenerative, and immunomodulatory abilities (5). However, although they can be obtained from third-party donors, the possibility of the persistence of implanted MSCs in recipient patients or the formation of ectopic tissues has hindered their application in bone injury healing (6). Additionally, other limitations for their application include the difficulty of maintaining optimal potency and viability during cell expansion and the method of administration to the patient (7). Recent studies have revealed that the potential of MSCs in tissue regeneration is linked to their paracrine activity, which partly depends on extracellular vesicles (EVs) derived from the secretome. Thus, the use of these EVs in regenerative medicine has been proposed as a cell-free therapeutic strategy (8).

EVs play an intercellular communication role and contain a wide variety of biologically active molecules, such as proteins, lipids, and different types of nucleic acids that can be relevant in the inflammatory response and tissue regeneration through signals transmitted to recipient cells (9). Depending on their size, EVs can be classified as microvesicles, exosomes, and apoptotic bodies. Specifically, exosomes range from 40 to 100 nm, have an endosomal origin, and are released by exocytosis from multivesicular bodies through plasma membranes (7). Thus, they can be administered intravenously and circulate through bodily fluids, such as blood, urine, or saliva (10). These exosomes seem to transmit the therapeutic effects of the originating cell while overcoming the limitations associated with the use of cells in regenerative medicine (7,9). They can be isolated from multiple bodily fluids such as semen, blood, urine, saliva, breast milk, amniotic fluid, ascitic or cerebrospinal fluid, and bile (11). The content of these circulating EVs depends on the organism's state, making them a source of biomarkers and factors that can even be used for therapeutic applications as an alternative to MSC-derived EVs. Indeed, using MSC-derived EVs involves manipulating *in vitro* cell cultures, which may cause loss of MSC properties and genetic instability when performed outside their natural niche (12). This can be partially avoided using stable and immortalized MSC lines obtained by genetic manipulation, for example, inducing the expression of human telomerase reverse transcriptase (hTERT). This procedure has produced MSCs with high proliferation and expansion capacity, maintaining their immunomodulatory, differentiation, and regenerative properties (13,14). Although various animal studies have shown that over time, immortalized MSCs do not transform into tumor cells, and therefore conclude that they can be considered safe for potential clinical use, these studies have certain limitations. Among them are the lack of clinical trials and the still unknown possibility of immortalized MSCs accumulating unwanted mutations after prolonged expansion periods. Therefore, it has been suggested that the use of these cells, both in cell therapy and in cell-free therapy based on the use of EVs, should be subject to strict controls during culture (15). This increases the complexity of the procedure due to the need to maintain stable MSC lines and the design of bioreactors for large-scale EV production. Therefore, it is interesting to study other alternative sources of regenerative EVs, such as circulating EVs in blood, which can be obtained without the need to expand and maintain cells in culture (16). Moreover, the amount of EVs obtained from plasma can be between 10 and more than 100 times higher than that obtained from cell cultures (17). One potential source is umbilical cord blood. It has a composition similar to that of adult bone marrow, but unlike this, it also contains a series of immunosuppressive cells, allowing it to reduce levels of inflammatory cytokines (18). In humans, it has been observed that human umbilical cord blood cell-derived extracellular vesicles (UCBEV) vs peripheral blood-derived derived extracellular vesicles from adults (peEV) have higher expression of miRNAs involved in pregnancy, leukemia suppression, inflammation inhibition, cell mobility, and nervous system development, as well as factors related to embryonic development (19,20), suggesting a high regenerative potential. As for peEV, several studies have shown their therapeu-

tic potential for the treatment of ischemic processes and wound healing (21). Thus, the objective of this study was to evaluate the potential effects of UCBEV and peEV on endothelial cell angiogenesis and MSC osteoblastic and adipogenic differentiation.

MATERIAL AND METHODS

EXTRACTION OF EXTRACELLULAR VESICLES FROM BLOOD PLASMA

After signing informed consent, healthy women without chronic pathologies and of adult age (between 26 and 31 years) underwent a single blood extraction. The umbilical cord blood was donated by mothers who met the same inclusion criteria as the healthy adults and also signed their participation consent.

Blood samples were centrifuged at 3000 rpm for 10 minutes to obtain plasma. One milliliter of plasma was passed through PURE-EVs size-exclusion chromatography columns (HansaBioMed Life Sciences Ltd.) using phosphate-buffered saline (PBS) as the vehicle. The 3 milliliters in which the EVs eluted were concentrated by ultrafiltration with 10 MWCO concentrators (Vivaspin 6 centrifugal concentrator, Sartorius) until a volume of 300-350 µl was obtained, which was stored at -20 °C until use. The concentration and size of the exosomes obtained from each sample were determined with a nanoparticle tracking analyzer (Nanosight NS300) based on "Nanoparticle Tracking Analysis" (NTA) technology at the University Institute of Nanochemistry (IUNAN) at the Universidad de Córdoba.

CHARACTERIZATION OF EVS BY WESTERN BLOT

EVs were characterized by Western blot. For this, 10 µg of each sample were loaded into an 8-16 % acrylamide gel (nUView Tris-Glycine Precast Gels, NuSeP) under denaturing conditions and separated by electrophoresis using the "Mini-Protean" system (Bio-Rad). Then, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using the Trans-Blot Transfer System (Bio-Rad). The membrane was blocked with a 5 % skim milk solution in TTBS buffer (20 nM Tril-CL pH 7.6, 150 mM NaCl, 0.05 % Tween) for 1 hour at room temperature and incubated overnight at 4 °C with primary antibodies anti-CD81 (25kDa, 1:500; ref.: 10630D), CD9 (25kDa, 1:1000; ref.: 10626D), and anti-CD63 (30-60 kDa, 1:1000; ref.: 10628D), all in 1 % milk with TTBS. Subsequently, the membrane was washed with TTBS and incubated for 1 hour with the secondary anti-mouse antibody (1:4000; ref.: 32430), in 2 % milk with TTBS. All antibodies used were from Invitrogen, ThermoFisher Scientific. Detection was performed using the chemiluminescent substrate Clarity Western ECL Substrate (Bio-Rad), and the images were acquired with the ChemiDoc™ XRS+ system (Bio-Rad) using the Image Lab 6.1 software from the same manufacturer.

MSC AND HUVEC CELL CULTURES

The MSCs used were isolated from cryopreserved mononuclear cells (Stemcell Technologies, Cologne, Germany) according to the protocol previously described by our group (22). They were expanded in minimum essential alpha medium (αMEM) from Biowest (Nuaillé, France), containing 2 mM of ultraglutamine (Biowest), 10 % fetal bovine serum (FBS; Gibco-Thermo Fisher Scientific), 100 U of penicillin, 0.1 mg of streptomycin/mL, and 1 ng/mL of fibroblast growth factor (FGF-2) from Sigma-Aldrich (Saint Louis, MO, USA). Umbilical vein endothelial cells (HUVEC) from Lonza (Basel, Switzerland) were grown in endothelial basal medium (EBM), with supplements and growth factors, known as endothelial growth medium (EGM) from Lonza. This contained 10 % FBS, hydrocortisone, gentamicin, human epidermal growth factor (hEGF), and bovine brain extract.

Both MSC and HUVEC cultures were incubated at 37 °C with 95 % humidity and 5 % CO_2. When they reached 90 % confluence, the cells were lifted with trypsin-EDTA (Gibco) and seeded in 12 (P12), 24 (P24), or 96-well (P96) plates for different experiments.

CELL VIABILITY ASSAY

Cell viability was determined using the 3-(4,5-dimethylthiazol-2)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) assay. MSCs and HUVECs were seeded in P96 plates at a density of 4000 and 8000 cells per well, respectively, in the corresponding culture medium for each cell type, as described earlier. After 24 hours, the medium was replaced with medium containing exosome-free FBS, supplemented with different concentrations of UCBEV or peEV (10×10^6, 20×10^6, 40×10^6, 80×10^6, and 160×10^6 particles/mL). After 72 hours, the medium was removed, and 50 µL per well of Dulbecco's modified Eagle medium (DMEM) without phenol red, supplemented with 1 mg of MTT/mL (both from Sigma-Aldrich), was added. After 2 hours of incubation, the medium was removed, and the formazan crystals produced were dissolved in isopropanol. The resulting solution's absorbance was measured at 570 nm, with the absorbance at 650 nm used as a reference, using a BioTek Instruments PowerWave XS microplate spectrophotometer (Winooski, VT, USA).

CELL MIGRATION ASSAY

Migration was studied using the Scratch Assay in P24 plates. In confluent cultures, a cell-free zone was generated using the tip of a P200 pipette, and different concentrations of UCBEV or peEV (10×10^6 and 160×10^6 particles/mL) were added to the medium. Cultures were maintained for up to 24 hours, and images were taken at different times using the IncuCyte Zoom Imaging System from Sartorius. Images at 18 and 15 hours of migration for MSC and HUVEC, respectively, were analyzed with ImageJ V1.53f51 software (NIH; Bethesda, MD, USA). The times were selected because, after these times, the cell-free zone was fully occupied, making it impossible to identify differences between treatments. Migration was quantified relative to the percentage of the initial area not occupied by cells.

DIFFERENTIATION OF MSCs INTO OSTEOBLASTS AND ADIPOCYTES

In MSC cultures at 60-80 % confluence, differentiation to osteoblasts or adipocytes was induced. Osteoblastic differentiation was maintained for 21 days in medium supplemented with 10 nM dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerol phosphate (Sigma-Aldrich), in the presence or absence of the different EV concentrations tested (10×10^6 and 160×10^6 particles/mL). On the other hand, differentiation into adipocytes was induced with 500 nM dexamethasone, 0.5 mM isobutylmethylxanthine, and 50 µM indomethacin (all from Sigma-Aldrich), maintained for 14 days, in the presence or absence of the different EV concentrations.

CYTOCHEMICAL STAINING

Alizarin red staining at 21 days of osteoblastic differentiation was used to visualize and quantify extracellular matrix mineralization. Cultures in P12 plates were fixed with 3.7 % formaldehyde for 10 minutes and then stained with 40 mM alizarin red in water, pH 4.15 (Sigma-Aldrich) for 10 minutes. The wells were then washed several times with 60 % isopropanol, dried, and visualized under an optical microscope. Alizarin red deposits were measured after elution with 10 % acetic acid, neutralization with 10 % ammonium hydroxide, and quantification by spectrophotometry at 405 nm absorbance of the resulting solution.

The formation of fat vesicles in cultures induced to differentiate into adipocytes was evaluated by Oil Red O staining. For this, cells were fixed with 3.7 % formaldehyde for 15 minutes, washed with 60 % isopropanol in water, and stained for 15-20 minutes with a 0.3 % Oil Red O solution (weight/volume) in 60 % isopropanol. The cells were then washed twice with distilled water, stained with hematoxylin, and images were taken, at least nine per well, using an optical microscope. Fat vesicle staining was quantified with ImageJ software (NIH), and values were normalized with the number of cells per image. Lipid accumulation in the cultures was expressed as: (Oil Red O stained area / number of cells).

RNA ISOLATION AND GENE EXPRESSION QUANTIFICATION

Samples for RNA isolation and subsequent analysis of adipogenesis and osteoblastogenesis marker genes were taken from MSC cultures 10 days after induction to differentiate into osteoblasts or adipocytes. RNA was isolated following the manufacturer's instructions using the NZY total RNA isolation kit (NZYTech, Lisbon, Portugal) and quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Then, 900 ng were retrotranscribed using the iScript cDNA synthesis kit from Bio-Rad (Hercules, CA, USA). Quantitative real-time polymerase chain reaction (RT-qPCR) tests were carried out in a CFX96 Connect (Bio-Rad). Each reaction contained 1 µL of cDNA, 10 pmol of each primer pair (Table I), and SensiFAST Sybr No-Rox Mix from Bioline (London, UK). The PCR amplification program included a cycle at 95 °C for 2 minutes (DNA denaturation and polymerase activation) and 44 amplification cycles: 95 °C for 5 seconds (DNA denaturation) and 65 °C for 30 seconds (hybridization and extension). Results were analyzed using the CFX Maestro V 2.3 software (Bio-Rad) to obtain threshold cycles (Ct). The POLR2A gene, encoding RNA polymerase II subunit A, was used as a constitutive gene, and relative expression vs control samples was expressed as arbitrary units calculated using the $2^{-(\Delta\Delta Ct)}$ method, where $\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (control sample); and ΔCt (sample) = Ct (gene of interest sample) – Ct (constitutive gene sample), and ΔCt (control sample) = Ct (gene of interest control sample) – Ct (constitutive gene control sample).

ANGIOGENESIS ASSAY IN HUVEC

To evaluate the effect of UCBEV and peEV on angiogenesis in HUVEC, a tube formation assay in Matrigel was performed. HUVEC cells were pretreated for 24 hours with different concentrations of UCBEV or peEV (10×10^6 and 160×10^6 particles/mL) in EGM medium + 10 % FBS without exosomes. For the angiogenesis assay, 10 µL of reduced growth factor Matrigel (Corning, NY, USA) at 4 °C was added to P96 microplates from Greiner Bio-One (Kremsmunster, Austria), allowed to gel at room temperature. Then, from each HUVEC culture pretreated with the different EVs, 15,000 cells per well were added, resuspended in 70 µl of EBM + 2 % FBS without exosomes, supplemented with the corresponding type and concentration of EV. The cells were maintained under culture conditions for 4 hours at 37 °C and 5 % CO_2.

Table I. Primers used for QRT-PCR

Gene	Sequence (5'-->3')	Product (bp)
Polymerase (RNA; DNA directed) II polypeptide A (POLR2A)	TTTTGGTGACGACTTGAACTGC CCATCTTGTCCACCACCTCTTC	125
Runt-related transcription factor 2 (RUNX2)	TGGTTAATCTCCGCAGGTCAC ACTGTGCTGAAGAGGCTGTTTG	143
Osterix (SP7)	AGCCAGAAGCTGTGAAACCTC AGCTGCAAGCTCTCCATAACC	163
Integrin-binding sialoprotein (IBSP)	AGGGCAGTAGTGACTCATCCG CGTCCTCTCCATAGCCCAGTGTTG	171
Collagen, type I, alpha 1 (COL1A1)	CGCTGGCCCCAAAGGATCTCCTG GGGGTCCGGGAACACCTCGCTC	263
Peroxisome proliferator activated receptor gamma 2 (PPARG2)	GCGATTCCTTCACTGATACACTG GAGTGGGAGTGGTCTTCCATTAC	136
Patatin Like Phospholipase Domain Containing 2 (ATGL)	CCAACACCAGCATCCAGTTCA ATCCCTGCTTGCACATCTCTC	102
Lipoprotein lipase (LPL)	AAGAAGCAGCAAAATGTACCTGAAG CCTGATTGGTATGGGTTTCACTC	113
Fatty-acid-binding protein 4 (FABP4)	TCAGTGTGAATGGGGATGTGAT TCTGCACATGTACCAGGACACC	162

After this period, images were taken using an optical microscope and analyzed with the Angiogenesis Analyzer extension of ImageJ software. As a negative control of angiogenesis, cultures maintained in unsupplemented medium without EVs were used, and as a positive control, the medium was supplemented with 30 ng/mL of the angiogenesis inducer fibroblast growth factor 2 (FGF-2).

STATISTICAL ANALYSIS

Data are expressed as the mean ± standard error of the mean (mean ± SEM). In all experiments, the number of replicates for each parameter studied was at least 3. Comparison between the different treatments was performed using the ANOVA test to detect significant changes, followed by a Tukey test to identify significant differences between pairs of treatments. Significant changes were considered for $p < 0.05$.

RESULTS

CHARACTERIZATION OF CIRCULATING EVs FROM UMBILICAL CORD BLOOD OR ADULT PLASMA

The size analysis of the UCBEV and peEV by Nanoparticle Tracking Analysis shows that most of the EVs obtained have a diameter ranging from 50 to 150 nm (Figs. 1 A and B). The concentration of particles per ml of umbilical cord plasma ranged from 0.7×10^{11} to 1.3×10^{11}, and for adult blood plasma, it ranged from 3.2×10^{11} to 1.1×10^{12}. On the other hand, the characterization of the protein expression of the markers CD81, CD9, and CD63, by Western blot, indicated that both types of EVs express these markers (Fig. 1C). These results show how, after processing the plasma samples from umbilical cord blood or adult blood, circulating EVs were obtained.

EFFECT OF UCBEV AND peEV ON CELL VIABILITY OF MSCs AND HUVECs

To evaluate the effect of UCBEV and peEV on the cell viability of MSC cultures, the cells were treated with different concentrations of EVs (10×10^6, 20×10^6, 40×10^6, 80×10^6, and 160×10^6 particles/mL) for 48 hours. The results showed that the viability was not significantly affected by any of the doses and types of EVs (Fig. 2A). Based on these results, in HUVEC cultures, the effect of the lowest and highest concentration of EVs used in the study on MSC viability (10×10^6 and 160×10^6 particles/mL) was evaluated. In this case, a significant increase in viability was observed when the cells were treated with the smaller concentration (10×10^6) of particles/mL of peEV (Fig. 2B).

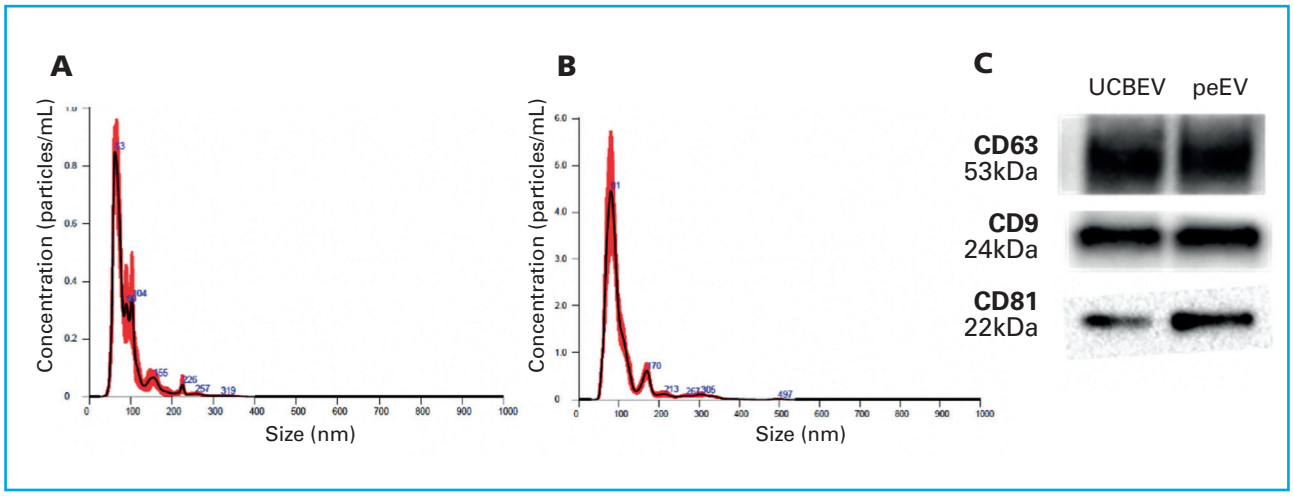

Figure 1. Quantification and characterization of circulating exosomes isolated from umbilical cord blood plasma (UCBEV) or peripheral blood plasma from healthy adults (peEV). A and B. Show the analysis of particle size distribution obtained from UCBEV and peEV, respectively. C. Shows the protein expression via Western blot of EV markers CD63, CD9, and CD81 in UCBEV and peEV.

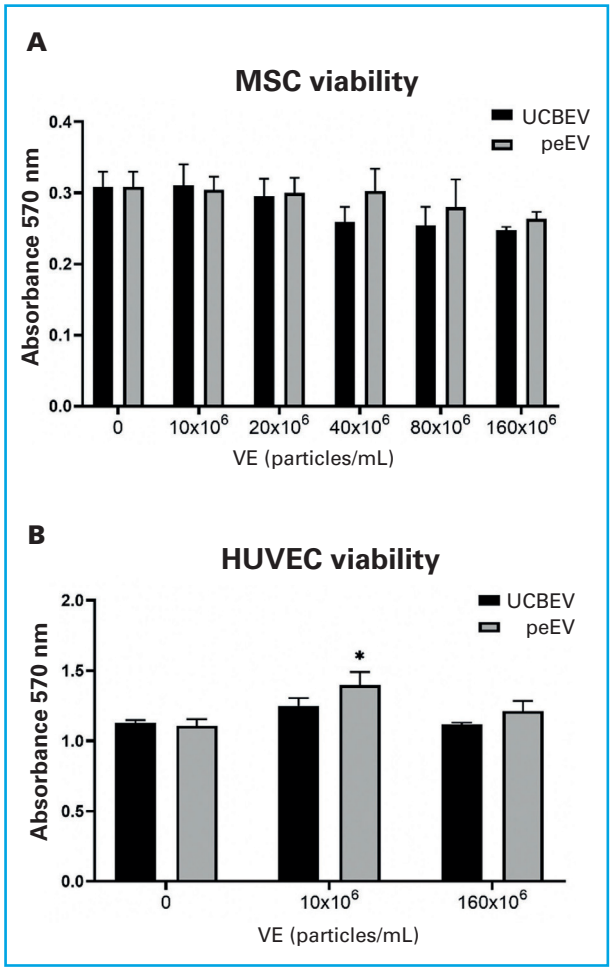

Figure 2. Quantification of cell viability in MSC (A) and HUVEC (B) treated for 72 hours with different concentrations of circulating UCBEV or peEV. *$p < 0.05$ compared to untreated cultures (control).

EFFECT OF UCBEV AND peEV ON MSC AND HUVEC MIGRATION

In the cell migration assay, the presence of 10×10^6 or 160×10^6 particles/mL of both types of EVs (UCBEV and peEV) reduced migration in MSC and HUVEC cultures, after 18 hours or 15 hours, respectively (Fig. 3). The decrease was more pronounced in the presence of peEV (Fig. 3).

EFFECT OF UCBEV AND peEV ON MSC DIFFERENTIATION

Osteogenic differentiation

The results of extracellular matrix mineralization after 21 days of osteogenic differentiation in MSCs induced to differentiate into osteoblasts in the presence of different concentrations of UCBEV and peEV, show that none of the concentrations used affected this mineralization (Figs. 4 A and B).

After 10 days of osteoblastic differentiation, the expression of osteoblastic marker genes, such as the *transcription factor RUNX2, osterix* (SP7), the integrin-binding sialoprotein (IBSP), and type I collagen alpha-1 (COL1A1), was also studied in these cultures. These genes code for two transcription factors responsible for osteogenic differentiation and proteins of the extracellular matrix. The results shown in figure 4C indicate that, like mineralization, treatment with the different types of EVs did not produce significant changes in the expression of these osteoblastic genes. Only in the expression of COL1A1, an increase was observed in cultures treated with the highest concentration of EVs (Fig. 4C).

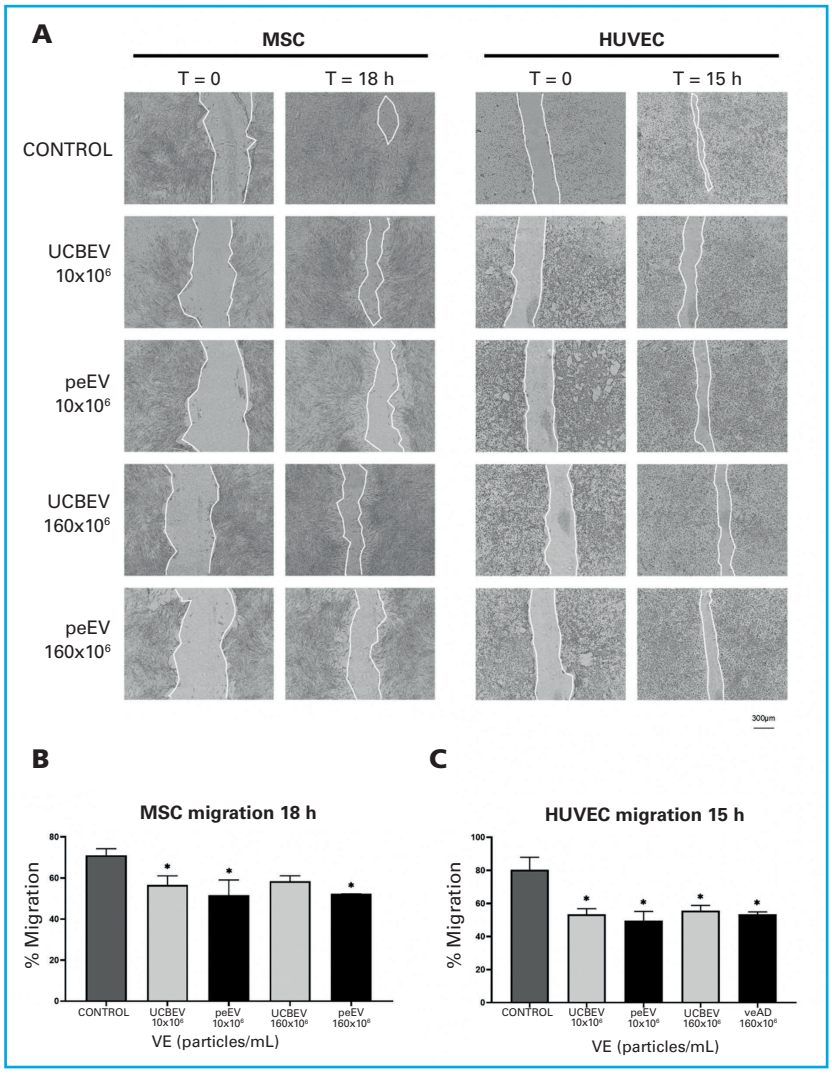

Figure 3. Effect of UCBEV and peEV on cell migration. A. Representative images of MSC or HUVEC cultures at time 0, after creating a cell-free line on the plate, and at 18 or 15 hours of culture, respectively, in the presence or absence of different concentrations of UCBEV or peEV. B. Quantification of the migration area percentage in MSC after 18 hours of treatment. C. Same as B, after 15 hours with HUVEC. *$p < 0.05$ compared to untreated cultures (control).

Adipogenic differentiation

In MSCs differentiated into adipocytes, the results of the Oil Red O staining analysis showed that the presence of UCBEV or peEV in the adipogenic medium reduced the formation of fat vesicles compared to the untreated cultures (Fig. 5B). Regarding the expression of the gene coding for the main transcription factor responsible for adipogenic differentiation, peroxisome proliferator-activated receptor gamma 2 (PPARG2), no significant differences were observed between the different treatments after 10 days of adipogenic differentiation. Changes were observed in the expression of genes involved in fatty acid metabolism, such as adipose triglyceride lipase (ATGL) and fatty acid-binding protein 4 (FABP4). The mRNA levels of ATGL decreased in cultures treated with 160 × 10^6 particles/mL of UCBEV, and the gene expression of FABP4 was inhibited with both concentrations of peEV used (Fig. 5C). The decrease in the expression of the genes ATGL and FABP4 may be related to the reduction in fat vesicle formation and different mechanisms of action of UCBEV and peEV on MSC cultures differentiated into adipocytes.

STUDY OF THE EFFECT OF UCBEV AND peEV ON ANGIOGENESIS

The results of the quantification of the total length of segments and tubular structures indicate that all EV treatments increased angiogenesis vs untreated HUVECs (Control -). This increase was more significant with UCBEV treatments (Figs. 6 B and C).

DISCUSSION

Regenerative medicine applied to bone has great potential for the treatment of pathologies such as osteoporosis, osteoarthritis, osteonecrosis, and trau-

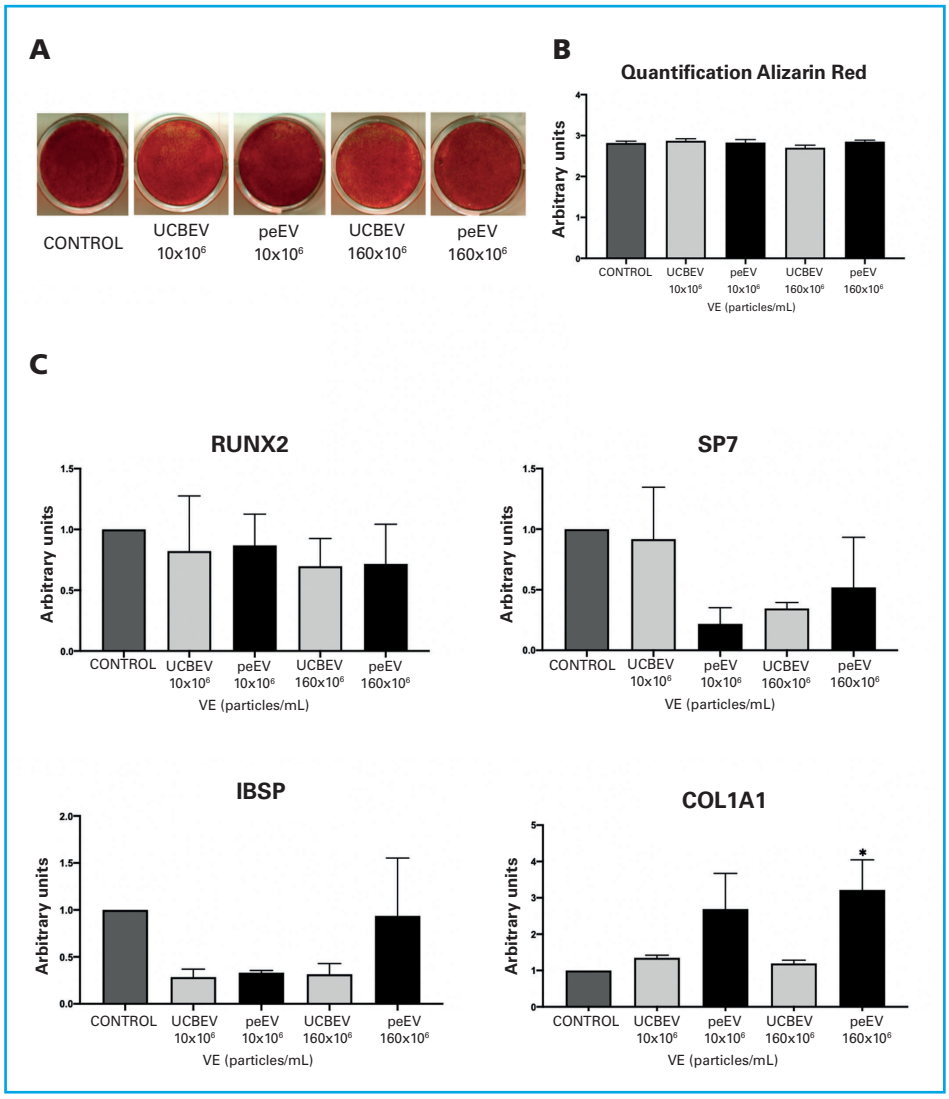

Figure 4. Study of the osteoblastic differentiation capacity of MSC treated with different concentrations of UCBEV and peEV. A. Representative images of Alizarin red staining for extracellular matrix mineralization in MSC cultures induced to osteoblasts and treated with different concentrations of UCBEV or peEV for 21 days. B. Quantification of Alizarin red staining. C. Gene expression of osteoblastic markers (RUNX2, SP7, IBSP, and COL1A1) in MSC cultures treated with UCBEV or peEV, 10 days after induction to differentiate into osteoblasts. *$p < 0.05$ compared to untreated cultures (control).

matic fractures. Although cell therapy has been widely evaluated in bone regeneration with promising results (23), it is currently considered that the use of EVs derived from MSCs as a cell-free therapeutic tool could avoid the drawbacks of producing and implanting progenitor cells for therapeutic purposes in bone (7). However, obtaining EVs derived from MSCs, even from immortalized cell lines, requires the establishment and maintenance of stable cell cultures, which increases the complexity of their isolation and requires suitable facilities for clinical use. Therefore, it is advisable to evaluate the potential therapeutic capacity of other sources of EVs that are easily accessible, abundant, and do not present significant ethical issues.

Thus, the aim of this work was to evaluate the effect of EVs derived from umbilical cord blood and from healthy adult individuals on processes related to bone regeneration, such as angiogenesis in endothelial cells and osteoblastic and adipogenic differentiation in bone marrow-derived MSCs.

Our results showed that both types of EVs did not significantly affect osteogenic differentiation but reduced adipogenesis in MSCs and increased angiogenesis in HUVECs. While UCBEV seem to favor angiogenesis more, and peEV may intervene more significantly in fat metabolism through inhibition of FABP4, we did not detect other major differences between both types of EVs. This could be partly because, in addition to nutrients and oxygen from the maternal blood, the umbilical vein blood also transports EVs from the mother (24). Therefore, besides fetal-origin exosomes, there would also be adult-origin exosomes.

The response to tissue damage requires a series of molecular and cellular events, including cell migration among others (25). According to the results obtained, migration of MSCs and HUVECs decreased, although with UCBEV, the reduction in cell migration was smaller than with peEV. These results suggest that the content of both types of EVs favored differentiation but reduced cell migration.

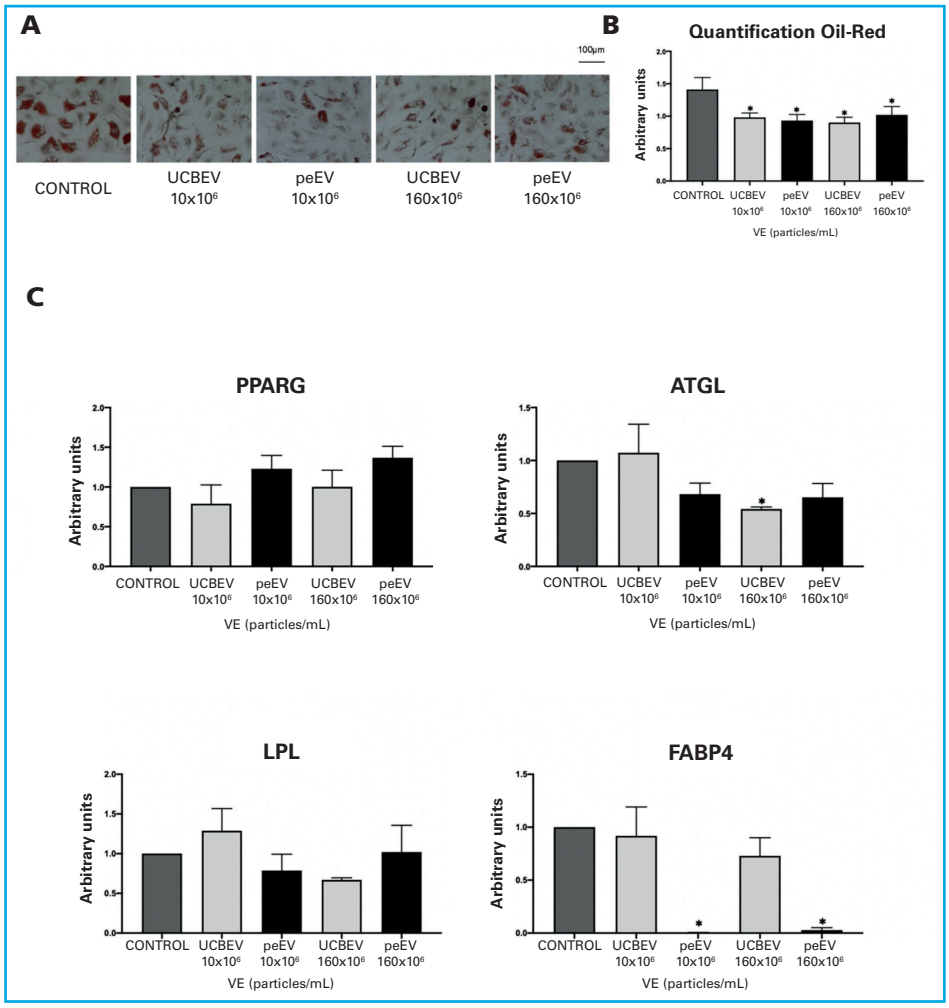

Figure 5. Study of the adipogenic differentiation capacity of MSC treated with different concentrations of UCBEV and peEV. A. Representative images of "Oil Red O" staining of fat vesicles in MSC cultures induced to adipocytes and treated with different concentrations of UCBEV or peEV for 14 days. B. Quantification of "Oil Red O" staining. C. Gene expression of adipogenic marker genes (PPARG, ATGL, LPL, and FABP4) in MSC cultures treated with UCBEV or peEV, 10 days after induction to differentiate into adipocytes. *$p < 0.05$ compared to untreated cultures (control).

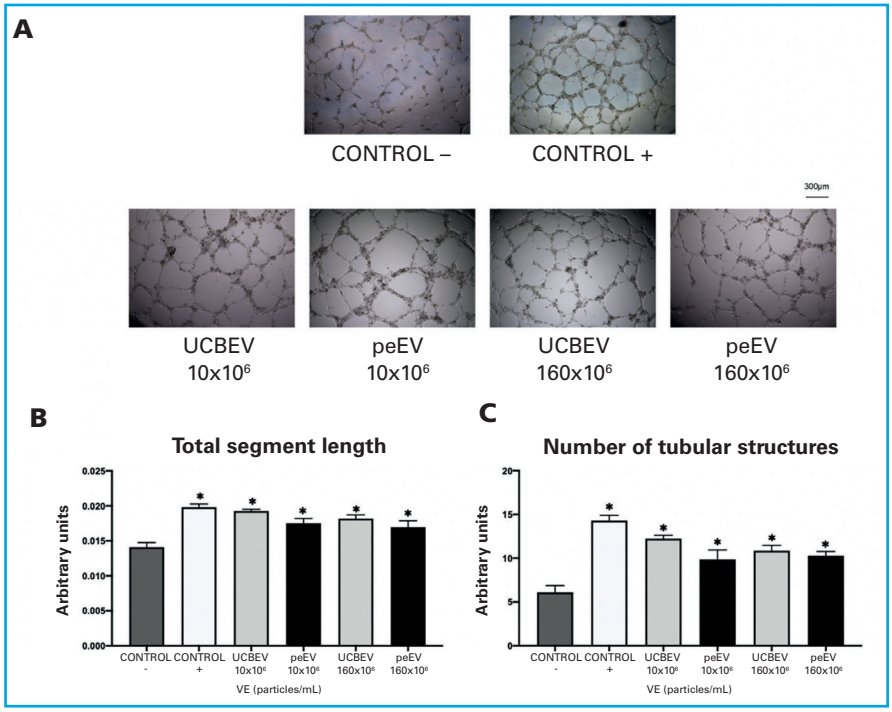

Figure 6. Study of angiogenesis in HUVEC cultures. A. Representative images of tubular structure formation in HUVEC cultures on matrigel and treated with different concentrations of UCBEV or peEV. The control (-) corresponds to untreated cultures, and the control (+) to cultures treated with bFGF (30 ng/mL) as an angiogenic factor. B and C. Graphical representation of the quantification in the angiogenesis assay of the total segment length and the number of tubular structures, respectively. *$p < 0.05$ compared to untreated cultures (control [-]).

This effect has been previously described by other authors, who showed that during osteogenic and chondrogenic differentiation of MSCs, migration decreased as differentiation progressed (26). Also, other authors have described that EVs derived from plasma of healthy individuals inhibit migration of microvascular endothelial cells (27).

Osteogenic differentiation of MSCs is a complex process regulated by various factors, such as the bone microenvironment, which significantly influences osteogenesis (28), and in which EVs participate, regulating different physiological aspects of stem cells (29). In this context, our results showed that the gene expression of COL1A1 increased in cultures treated with peEV, which could favor mineralization (30). However, in the expression of other osteoblastic genes, no significant changes were observed with treatments of peEV or UCBEV. This can be related to the fact that none of the EVs evaluated affected the mineralization of MSCs differentiated into osteoblasts. Induction of bone formation by EVs derived from MSCs has been observed in numerous previous studies (31). However, the application of exosomes derived from plasma of healthy adolescents on undifferentiated MSCs did not significantly affect osteogenic differentiation, although when treated with primary osteoblast cultures, an increase in alkaline phosphatase (ALPL) activity was observed (7). However, in that study, mineralization was not studied, so the possible effect of those exosomes on the final maturation of the treated osteoblasts cannot be concluded. In another study, it was shown that treatment of MSC cultures derived from bone marrow with EVs from umbilical cord blood plasma increased mineralization (32). The differences with the results obtained in the present study could be due to the concentration of exosomes used (which cannot be compared as it is expressed in µg/mL) and the MSCs being derived from mice and not humans.

Regarding adipogenic differentiation, treatment with the evaluated circulating EVs led to a decrease in fat vesicle formation. This was accompanied by a decrease in the expression of the ATGL gene in cultures treated with UCBEV and of FABP4 in peEV, involved in fat metabolism and fat vesicle formation (33,34). It is interesting to note that the expression of the PPARG gene was not affected by the treatments, suggesting that the EVs used may affect more the maturation than the early differentiation of adipocytes.

The fact that each of the evaluated circulating EV types affected the expression of different adipogenic genes suggests differences in their cargo, which could include miRNA content. It has been demonstrated that the expression of FABP4 can be inhibited by several miRNAs, such as miR-369-5p and miR-455 (35), while ATGL can be inhibited by hsa-miR-214-3p (36). Therefore, in subsequent studies, it would be interesting to determine the cargo differences between UCBEV and peEV to identify possible mechanisms of action on MSC differentiation. It should also be emphasized that, regarding the effect of circulating EVs on adipogenesis, it is important to consider the nature and health of the donors. Thus, circulating exosomes from obese adolescents favor adipogenic differentiation more than osteogenic differentiation compared to those derived from healthy adolescents with normal weight (31).

It has been reported that the increase in adiposity in bone marrow during aging is caused by changes in the marrow microenvironment, which favor differentiation of MSCs into adipocytes rather than osteoblasts (37). Therefore, although our results do not show an effect of UCBEV and peEV on osteoblastogenesis, they have shown their ability to decrease adipogenesis. Therefore, the application of these vesicles could prevent the increase in adiposity in bone marrow and consequently favor bone formation through a potential increase in osteoprogenitors. This is supported by studies showing that intravenous injection of EVs from umbilical cord blood in old mice for two months, once a week, reduced age-related bone loss, stimulating bone formation and inhibiting bone resorption (32).

Blood vessel formation is essential in regenerative processes to provide nutrients, oxygen, and facilitate the arrival of progenitor and immune cells. Our results indicate that the evaluated circulating EVs increased angiogenesis in HUVECs. Other authors have shown an angiogenic effect of UCBEV in pigs (38) and other animal models. *In vitro* studies have shown that EVs derived from umbilical cord blood and from the mother increase angiogenesis in human microvascular endothelial cells (HMEC) (39) and HUVECs (40). Exosomes derived from serum of healthy humans aged 20-30 years also have a proangiogenic effect through the inhibition of inflammation in macrophages. Thus, local application of these exosomes along with bone grafts favored bone regeneration in a model of mandibular bone defects in rats through reduced inflammation and increased angiogenesis (41). Therefore, our results are in line with these studies and support that both healthy adult and umbilical cord blood could represent an abundant source of EVs for therapeutic purposes. Potential therapeutic applications would include those related to bone formation due to their effects on MSC differentiation and the induction of angiogenesis.

CONCLUSIONS

In conclusion, both types of EVs did not affect mineralization in MSCs differentiated into osteoblasts but decreased adipogenesis in MSCs and increased angiogenesis in HUVECs. These findings suggest that both types of EVs, from abundant sources without significant ethical issues and easy to isolate, have high potential in regenerative medicine applied to bone, inhibiting bone marrow adiposity and favoring angiogenesis.

REFERENCES

1. Schmidt-Bleek K, Petersen A, Dienelt A, Schwarz C, Duda GN. Initiation and early control of tissue regeneration – bone healing as a model system for tissue regeneration. Expert Opin Biol Ther 2014;14(2):247-59. DOI: 10.1517/14712598.2014.857653
2. Marzona L, Pavolini B. Play and players in bone fracture healing match. Clin Cases Miner Bone Metab 2009;6(2):159-62.
3. Yoo JU, Johnstone B. The role of osteochondral progenitor cells in fracture repair. Clin Orthop Relat Res 1998;(355 Suppl):S73-81. DOI: 10.1097/00003086-199810001-00009
4. Kirkham AM, Bailey AJM, Tieu A, Maganti HB, Montroy J, Shorr R, et al. MSC-Derived Extracellular Vesicles in Preclinical Animal Models of Bone Injury: A Systematic Review and Meta-Analysis. Stem Cell Rev Rep 2022;18(3):1054-66. DOI: 10.1007/s12015-021-10208-9
5. Stanovici J, Le Nail LR, Brennan MA, Vidal L, Trichet V, Rosset P, et al. Bone regeneration strategies with bone marrow stromal cells in orthopaedic surgery. Curr Res Transl Med 2016;64(2):83-90. DOI: 10.1016/j.retram.2016.04.006
6. Prockop DJ, Brenner M, Fibbe WE, Horwitz E, Le Blanc K, Phinney DG, et al. Defining the risks of mesenchymal stromal cell therapy. Cytotherapy 2010;12(5):576-8. DOI: 10.3109/14653249.2010.507330
7. Torrecillas-Baena B, Pulido-Escribano V, Dorado G, Gálvez-Moreno MÁ, Camacho-Cardenosa M, Casado-Díaz A. Clinical Potential of Mesenchymal Stem Cell-Derived Exosomes in Bone Regeneration. J Clin Med 2023;12(13):4385. DOI: 10.3390/jcm12134385
8. Marolt Presen D, Traweger A, Gimona M, Redl H. Mesenchymal Stromal Cell-Based Bone Regeneration Therapies: From Cell Transplantation and Tissue Engineering to Therapeutic Secretomes and Extracellular Vesicles. Front Bioeng Biotechnol 2019;7:352. DOI: 10.3389/fbioe.2019.00352
9. Park KS, Bandeira E, Shelke GV, Lässer C, Lötvall J. Enhancement of therapeutic potential of mesenchymal stem cell-derived extracellular vesicles. Stem Cell Res Ther 2019;10(1):288. DOI: 10.1186/s13287-019-1398-3
10. Di C, Zhang Q, Wang Y, Wang F, Chen Y, Gan L, et al. Exosomes as drug carriers for clinical application. Artif Cells Nanomed Biotechnol 2018;46(sup3):S564-70. DOI: 10.1080/21691401.2018.1501381
11. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 2013;200(4):373-83. DOI: 10.1083/jcb.201211138
12. Sohni A, Verfaillie CM. Mesenchymal Stem Cells Migration Homing and Tracking. Stem Cells International 2013;2013(1):130763. DOI: 10.1155/2013/130763
13. Zhang Y, Liu J, Mo Y, Chen Z, Chen T, Li Y, et al. Immortalized Mesenchymal Stem Cells: A Safe Cell Source for Cellular or Cell Membrane-Based Treatment of Glioma. Stem Cells International 2022;2022(1):6430565. DOI: 10.1155/2022/6430565
14. Chen Q, Jin M, Wang S, Wang K, Chen L, Zhu X, et al. Establishing an hTERT-driven immortalized umbilical cord-derived mesenchymal stem cell line and its therapeutic application in mice with liver failure. J Tissue Eng 2023;14. DOI: 10.1177/20417314231200328
15. Yamaguchi N, Horio E, Sonoda J, Yamagishi M, Miyakawa S, Murakami F, et al. Immortalization of Mesenchymal Stem Cells for Application in Regenerative Medicine and Their Potential Risks of Tumorigenesis. Int J Mol Sci 2024;25(24):13562. DOI: 10.3390/ijms252413562
16. De Boer HC, Hovens MM, Van Oeveren-Rietdijk AM, Snoep JD, De Koning EJP, Tamsma JT, et al. Human CD34+/KDR+ cells are generated from circulating CD34+ cells after immobilization on activated platelets. Arterioscler Thromb Vasc Biol 2011;31(2):408-15. DOI: 10.1161/ATVBAHA.110.216879
17. Dong L, Zieren RC, Horie K, Kim CJ, Mallick E, Jing Y, et al. Comprehensive evaluation of methods for small extracellular vesicles separation from human plasma, urine and cell culture medium. J Extracell Vesicles 2020;10(2):e12044. DOI: 10.1002/jev2.12044
18. Liu J, Sun W, Liu C, Na Q. Umbilical Cord Blood-Derived Exosomes in Maternal-Fetal Disease: a Review. Reprod Sci 2023;30(1):54-61. DOI: 10.1007/s43032-022-00879-1
19. Cleys ER, Halleran JL, McWhorter E, Hergenreder J, Enriquez VA, da Silveira JC, et al. Identification of microRNAs in exosomes isolated from serum and umbilical cord blood, as well as placentomes of gestational day 90 pregnant sheep. Mol Reprod Dev 2014;81(11):983-93. DOI: 10.1002/mrd.22420
20. Huang S, Tang Z, Wang Y, Chen D, Li J, Zhou C, et al. Comparative profiling of exosomal miRNAs in human adult peripheral and umbilical cord blood plasma by deep sequencing. Epigenomics 2020;12(10):825-42. DOI: 10.2217/epi-2019-0213
21. de Boer C, Davies NH. Blood derived extracellular vesicles as regenerative medicine therapeutics. Biochimie 2022;196:203-15. DOI: 10.1016/j.biochi.2021.10.009
22. Casado-Díaz A, Santiago-Mora R, Jiménez R, Caballero-Villarraso J, Herrera C, Torres A, et al. Cryopreserved human bone marrow mononuclear cells as a source of mesenchymal stromal cells: application in osteoporosis research. Cytotherapy 2008;10(5):460-8. DOI: 10.1080/14653240802192644
23. Lin H, Sohn J, Shen H, Langhans MT, Tuan RS. Bone marrow mesenchymal stem cells: Aging and tissue engineering applications to enhance bone healing. Biomaterials 2019;203:96-110. DOI: 10.1016/j.biomaterials.2018.06.026
24. Nakahara A, Nair S, Ormazabal V, Elfeky O, Garvey CE, Longo S, et al. Circulating Placental Extracellular Vesicles and Their Potential Roles During Pregnancy. Ochsner J 2020;20(4):439. DOI: 10.31486/toj.20.0049
25. Velnar T, Bailey T, Smrkolj V. The wound healing process: an overview of the cellular and molecular mechanisms. J Int Med Res 2009;37(5):1528-42. DOI: 10.1177/147323000903700531
26. Sliogeryte K, Thorpe SD, Lee DA, Botto L, Knight MM. Stem cell differentiation increases membrane-actin adhesion regulating cell blebability, migration and mechanics. Sci Rep 2014;4:7307. DOI: 10.1038/srep07307
27. Ramakrishnan DP, Hajj-Ali RA, Chen Y, Silverstein RL. Extracellular Vesicles Activate a CD36-Dependent Signaling Pathway to Inhibit Microvascular Endothelial Cell Migration and Tube Formation. Arterioscler Thromb Vasc Biol 2016;36(3):534-44. DOI: 10.1161/ATVBAHA.115.307085
28. Li J, Liu X, Zuo B, Zhang L. The Role of Bone Marrow Microenvironment in Governing the Balance between Osteoblastogenesis and Adipogenesis. Aging Dis 2016;7(4):514-25. DOI: 10.14336/AD.2015.1206
29. Hou Y, Liu Y, Liang S, Ding R, Mo S, Yan D, et al. The novel targetexosoms derived from M2 macrophage. Int Rev Immunol 2021;40(3):183-96. DOI: 10.1080/08830185.2020.1800687

30. Veis A, Dorvee JR. Biomineralization mechanisms: a new paradigm for crystal nucleation in organic matrices. Calcif Tissue Int 2013;93(4):307-15. DOI: 10.1007/s00223-012-9678-2
31. Peruzzi B, Urciuoli E, Mariani M, Chioma L, Tomao L, Montano I, et al. Circulating Extracellular Vesicles Impair Mesenchymal Stromal Cell Differentiation Favoring Adipogenic Rather than Osteogenic Differentiation in Adolescents with Obesity. Int J Mol Sci 2023;24(1):447. DOI: 10.3390/ijms24010447
32. Hu Y, Xu R, Chen CY, Rao SS, Xia K, Huang J, et al. Extracellular vesicles from human umbilical cord blood ameliorate bone loss in senile osteoporotic mice. Metabolism 2019;95:93-101. DOI: 10.1016/j.metabol.2019.01.009
33. Kershaw EE, Schupp M, Guan HP, Gardner NP, Lazar MA, Flier JS. PPARγ regulates adipose triglyceride lipase in adipocytes in vitro and in vivo. Am J Physiol Endocrinol Metab 2007;293(6):E1736-45. DOI: 10.1152/ajpendo.00122.2007
34. Qian SW, Li X, Zhang YY, Huang HY, Liu Y, Sun X, et al. Characterization of adipocyte differentiation from human mesenchymal stem cells in bone marrow. BMC Dev Biol 2010;10:47. DOI: 10.1186/1471-213X-10-47
35. Tang W, Chen O, Yao F, Cui L. miR-455 targets FABP4 to protect human endometrial stromal cells from cytotoxicity induced by hydrogen peroxide. Mol Med Rep 2019;20(6):4781-90. DOI: 10.3892/mmr.2019.10727
36. Li Y, Yuan S, Wu W, Zhou J, Zhang P, Li D, et al. The hsa-miR-214-3p/ATGL axis regulates aberrant lipolysis to promote acute myeloid leukemia progression via PPARα in vitro. Biochem Biophys Res Commun 2022;608:73-81. DOI: 10.1016/j.bbrc.2022.03.140
37. Ambrosi TH, Scialdone A, Graja A, Gohlke S, Jank AM, Bocian C, et al. Adipocyte Accumulation in the Bone Marrow during Obesity and Aging Impairs Stem Cell-Based Hematopoietic and Bone Regeneration. Cell Stem Cell 2017;20(6):771-784.e6. DOI: 10.1016/j.stem.2017.02.009
38. Luo J, Fan Y, Shen L, Niu L, Zhao Y, Jiang D, et al. The Pro-angiogenesis Of Exosomes Derived From Umbilical Cord Blood Of Intrauterine Growth Restriction Pigs Was Repressed Associated With MiRNAs. Int J Biol Sci 2018;14(11):1426-36. DOI: 10.7150/ijbs.27029
39. Hu Y, Rao SS, Wang ZX, Cao J, Tan YJ, Luo J, et al. Exosomes from human umbilical cord blood accelerate cutaneous wound healing through miR-21-3p-mediated promotion of angiogenesis and fibroblast function. Theranostics 2018;8(1):169-84. DOI: 10.7150/thno.21234
40. Jia L, Zhou X, Huang X, Xu X, Jia Y, Wu Y, et al. Maternal and umbilical cord serum-derived exosomes enhance endothelial cell proliferation and migration. FASEB J 2018;32(8):4534-43. DOI: 10.1096/fj.201701337RR
41. Xiang X, Pathak JL, Wu W, Li J, Huang W, Wu Q, et al. Human serum-derived exosomes modulate macrophage inflammation to promote VCAM1-mediated angiogenesis and bone regeneration. J Cell Mol Med 2023;27(8):1131-43. DOI: 10.1111/jcmm.17727