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Original Article

Mesenchymal Stem Cells and Their Derived Exosomes Mitigated Hepatic Cirrhosis in Rats by Altering the Expression of miR-23b and miR-221

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Abstract

Background: The therapeutic effect of mesenchymal stem cells (MSCs) in liver cirrhosis is limited by their entrapment in the pulmonary vessels. Thus, the use of MSC-derived exosomes has become a promising strategy. The current work aimed to compare the role of human umbilical cord blood-MSCs (hUCB-MSCs) and their derived exosomes in the alleviation of liver cirrhosis focusing on the role of miR-23b and miR-221 and their direct effectors in inflammatory and autophagic pathways.

Methods: Rats were divided into six groups: normal controls (negative control), liver cirrhosis group (positive control), liver cirrhotic rats that received conditioned media, liver cirrhotic rats that received hUCB-MSCs, cirrhotic rats that received exosomes, and cirrhotic rats that received both hUCB-MSCs and exosomes. The messenger RNA expression of *transforming growth factor-β (TGF-β)*, *Matrix metalloproteinase 9 (MMP 9)*, *fibronectin*, *collagen type-1 (col1)*, *alpha-smooth muscle actin (α-SMA)*, *Suppressor of Mothers Against Decapentaplegic (SMAD) 2* and *7*, *Beclin*, *P62*, and *light chain 3* (*LC3)* were evaluated by quantitative real-time polymerase chain reaction. Immunohistochemical staining for Beclin, P62, and LC3 was performed.

Results: The treatment of cirrhotic rats with hUCB-MSCs, exosomes, or the combination of them significantly downregulated *miRNA-221*, *fibronectin, collagen I*, *α-SMA*, *Smad2* (P<0.001, for each), and *P62* (P=0.032, P<0.001, P<0.001, respectively). Additionally, the treatment of cirrhotic rats with hUCB-MSCs, exosomes, or the combination of them significantly upregulated *mTOR*, *Beclin*, *LC3*, and *Smad7 (*P<0.001, for each) and *miRNA-23* (P=0.021, P<0.001, P<0.001, respectively).

Conclusion: hUCB-MSCs and their derived exosomes ameliorated liver cirrhosis by anti-inflammatory and anti-fibrotic effects besides modulation of autophagy. The exosomes had a better improvement effect either alone or combined with hUCB-MSCs, as proved by improvement in liver function tests, and molecular, histopathological, and immunohistochemical profiles.

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Keywords ● Stem cells ● Exosomes ● Liver cirrhosis ● Gene expression ● microRNAs

What's Known

- The therapeutic effect of human umbilical cord blood-mesenchymal stem cells is possibly mediated by their exosomes.
- Exosomes support the therapeutic potency of stem cells by mediating intercellular communication and transporting paracrine factors.

What's New

- Exosomes improved the biochemical and histological profile of liver cirrhosis.
- Exosomes have anti-fibrotic and anti-inflammatory effects and modulate autophagy.

Introduction

Liver cirrhosis is caused by an excessive new extracellular matrix (ECM) deposition in response to injury. It also involves

the activation of hepatic stellate cells (HSCs), which are liver-specific pericytes found in the Disse space of the subendothelial layer. In the liver, HSCs transdifferentiate into fibrogenic myofibroblasts in response to inflammatory cytokines such as transforming growth factor (TGF)-β, which is also responsible for epithelial-to-mesenchymal transitions (EMT). This transdifferentiation leads to increased ECM synthesis and the expression of alpha-smooth muscle actin (α-SMA). Thus, TGF-β activation promotes ECM formation while inhibiting ECM degradation, hastening the progression of liver fibrosis. In liver cirrhosis, HSCs are the primary generator of collagen and other ECM proteins. HSCs transform from dormant cells to proliferative, α-SMA-producing myofibroblast-like cells with enhanced collagen synthesis after prolonged liver injury.[1]

TGF-β1 is activated by SMADs, which are transducer proteins. Smad6 and Smad7 mediate TGF-β1/SMAD signaling pathway negative feedback. Smad7 overexpression inhibits collagen expression and HSC cell proliferation.[2]

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can self-renew, differentiate into multiple lineages, and reduce inflammation. Previous research has shown that rat bone marrow-MSCs transplantation can reduce collagen accumulation and improve liver function.[3] According to a previous study, paracrine factors derived from MSCs, rather than hepatic differentiation, are responsible for their beneficial effects in the treatment of liver diseases.[4] However, the therapeutic effect is limited by stem cell trapping in the pulmonary vessels.[5] As a result, using MSC-derived exosomes as cell-free paracrine mediators has emerged as a promising strategy for treating a variety of diseases.[6] They are less complex and smaller than their parent cells, making them easier to produce and store. They are also devoid of viable cells and pose no risk of tumor formation. Furthermore, since they contain fewer membrane-bound proteins than their parent cells, they are less immunogenic.[7]

Exosomes are extracellular vesicles that range in size from 30 to 150 nm and are secreted by all cell types. They deliver functional microRNAs (miRNAs), messenger RNAs (mRNAs), proteins, and cytokines.[8] Exosomes support MSC therapeutic potency by mediating intercellular communication and transporting paracrine factors during angiogenesis, tissue regeneration, and immune regulation. Exosome membranes contain a high concentration of cholesterol, sphingomyelin, ceramide, and lipid raft proteins.[9]

A previous study showed that exosomes reduced liver inflammation, collagen deposition, and EMT. They also enhanced the regeneration of the hepatocytes and decreased oxidant status.[10] They also reduced necrotic areas in the liver and increased anti-inflammatory cytokines. Furthermore, they inhibited HSC activation *via* the Wnt/-catenin pathway.[11]

miR-23b regulates normal physiological function, cell differentiation, and cellular immunity. Many transcription factors affect miR-23b expression. Moreover, miR-23b is a diagnostic and prognostic marker of many diseases, including tumors and autoimmune diseases.[12] miR-221 is involved in the progression of hepatic diseases and is elevated in the liver tissues of HCC patients. Previous research has confirmed the role of miR-221 in autophagy regulation.[13]

This study aimed to investigate the potential role of exosomes derived from hUCB-MSCs in the treatment of liver cirrhosis via altering the expression of miR-23b and miR-221 and their effector molecules regulating hepatic fibrosis and autophagy.

Materials and Methods

Animals

This experimental study was performed in Zagazig, Egypt, 2023. All animal procedures were performed under the ethical guidelines of the Institutional Animal Care and Use Committee of Zagazig University, Zagazig, Egypt (ZU-IACUC/3/F/24/2023) in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals 8th Edition, 2011.[14] Six-week-old Wistar rats of either sex (160-200 g body weight) were purchased from Zagazig University's Faculty of Veterinary Medicine in Zagazig, Egypt. Rats were kept under constant temperature (25 °C), humidity (50%-70%), and a 12:12-hour light-dark cycle. Throughout the experiments, all rats had free access to standard chow and tap water; they were given 7 days to acclimate before the experiments. Animal experiments were carried out in the Clinical Chemistry and Stem Cell Laboratories, Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Zagazig University in collaboration with the Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.

Experimental Cirrhosis Model

Rats were randomly divided into six groups 10 rats in each. Group 1 included rats injected with phosphate-buffered saline (PBS) as the control group (0.2 mL PBS was injected intravenously via the tail vein as a single dose), group 2 included rats which received

carbon tetrachloride (CCl4) to induce liver cirrhosis, group 3 included rats which received CCl4 plus the injection of conditioned media (culture media containing biologically active components obtained from previously cultured stem cells), group 4 included rats which received CCl4 plus the injection of hUCB-MSCs, group 5 included rats which received CCl4 plus the injection of exosomes derived from hUCB-MSCs and group 6 included rats which received CCl4 plus the injection of both hUCB-MSCs and exosomes. Rat liver cirrhosis was induced by intraperitoneal administration of CCl4 (50% CCl4 0.1 mL/100 g [dissolved in oil]) twice a week for 8 weeks.[15] MSCs and MSCs-derived exosomes were stained with PKH-26 according to the manufacturer's instructions (Cat. No. PKH26GL, Sigma Aldrich, USA) and administered intraperitoneally at a dose of 2×10^7 dissolved in 0.2 mL PBS.[16] Three days after hUCB-MSCs and their derived exosomes administration, a rat from the MSCs-treated group and a rat from the exosomes-treated groups were sacrificed, and their livers were collected in 10% neutral formalin buffer to detect the homing of both hUCB-MSCs and exosomes.

After 4 weeks of injection of MSCs and/or their derived exosomes, the remaining rats of all groups were sacrificed, and blood and liver samples were collected. The serum was separated, aliquoted, and stored at -80 °C, and liver tissues were divided into two parts. One part was collected on 1 mL TRIzol (Cat. No. 15596026, Invitrogen, CA, USA) and stored at -80 °C for total RNA extraction. The second part was collected in 10% neutral formalin buffer for histopathological and immunohistochemical studies.

Isolation, Purification, and Characterization of MSCs

All pregnant women who agreed to donate their cord blood signed written informed consent forms. After delivery, cord blood was collected from five donors using a 50 mL sterile falcon tube and heparin as an anticoagulant. The blood was then allowed to clot for 2~4 h at room temperature. Cord blood was diluted with phosphate-buffered saline (PBS) (Cat. No. AM9625, Lonza, Basel, Switzerland) 1:1. The separation of mononuclear cells was performed by density gradient centrifugation using Ficoll (Lonza, Basel, Switzerland). The diluted cord blood was layered on Ficoll gently and slowly with the ratio of diluted cord blood and Ficoll 1:1. Centrifugation at room temperature was performed at 360 ×g for 20 min. After centrifugation, subsequent collection of the interface of the mononuclear cells was performed with subsequent wash twice by PBS before resuspension in complete culture media. The MSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS) at a density of 1×10^6 cells/mL. The non-adherent cells were discarded after 48 hours. Then, culture medium was replaced every 3 days. Cells were passaged using 0.05% trypsin-ethylene diamine tetra-acetic acid (EDTA) (Lonza, Basel, Switzerland) when the confluence level reached 80%. Fluorescence-activated cell sorting (FACS) was used to characterize the MSC markers at passage 3, including CD34, CD45, CD11b, CD44, CD105, CD90, CD73, and CD106.

Total Exosome Extraction from hUCB-MSCs

First, cultivation of hUCB-MSCs was performed in media without FBS for 12 hours. Then, centrifugation was performed to remove cells. The obtained volume of cell-free culture media supernatant was mixed with a half volume of the Total Exosome Isolation reagent (Cat. No. 4478359, Invitrogen, Thermo Fisher Scientific Inc., Massachusetts, USA). After an overnight incubation at 6 °C, centrifugation was performed to obtain pellets containing the exosomes.

Flow Cytometer Analysis of hUCB-MSCs and Exosomes

MSCs and the supernatant of exosome culture were pelleted at 300 ×g for 5 min and resuspended to a final volume of 1 mL of PBS. Filtered cells were dispensed to 100 µL aliquots (Lonza, Basel, Switzerland). MSCs were incubated with MSC-markers CD90FITC, CD73PE, CD34PE, CD45FITC, CD105 FITC, CD11b PE, CD44 FITC, and CD106 Peridinin-Chlorophyll-Protein (PerCP), while exosomes were incubated with CD9PE, CD63 PE and CD81 PerCP (Becton-Dickinson Biosciences, San Jose, CA, USA). The acquisition was done by 20,000 events.

Electron Microscopy for Exosomes

Transmission electron microscopy (TEM) was performed to detect the ultrastructure of the hUCB-MSCs-derived exosomes. The obtained exosome pellets were suspended in 2.5% glutaraldehyde (Cat. No. 104239, Merck KGaA, Darmstadt, Germany), 2% paraformaldehyde (Cat. No. 30525-89-4, Sigma-Aldrich Co.), and 0.1 M cacodylate buffer (Cat. No. J60367. AE, Thermo Fisher Scientific Inc., Massachusetts, USA). After overnight incubation, fixation with

1% osmium tetroxide (Cat. No. 20816-12-0, Sigma-Aldrich Co., Darmstadt, Germany) was performed. Ethanol and propylene oxide were used for sample dehydration. Agar 100 resin (AGR1031, Agar Scientific Ltd, Stansted, UK) was used to prepare blocks. Transmission electron microscope (TEM) JEM 1200EX (Oxford, UK) was used for examination of the sections.[17]

Liver Function Test

The alanine transaminase (ALT) (Ref: MD41274), aspartate transaminase (AST) (Ref: MD41264), alkaline phosphatase (ALP) (Ref: MD41233), and albumin (Ref: TK1001020) were analyzed using a semi-automatic biochemical analyzer (Sunostik, China). The kits were provided by Spinreact (Spain).

Quantitative Real-time Polymerase Chain Reaction (RT-PCR)

Total RNA samples were extracted using TRIzol (Cat. No. 15596026, Invitrogen, CA, USA), as directed by the manufacturer. The harvested RNA was run through gel electrophoresis and measured using Nanodrop spectrophotometry (ND 1000-NanoDrop®, Thermo Fisher Scientific Inc., Massachusetts, USA) to determine its quality and integrity. The complementary DNA (cDNA) was then synthesized from 1 µg RNA using a high-capacity cDNA reverse transcription kit according to the manufacturer's instructions (Cat. No. 4368814, Applied Biosystem, Thermo Fisher Scientific Inc., Massachusetts, USA). miRCURY LNA RT kit (Cat. No. 339340, Qiagen, Hilden, Germany) was used for cDNA synthesis from miRNA. Real-time quantitative polymerase chain reaction (RT-PCR) analysis was performed using Rotor-Gene Q 2 Plex (Qiagen, Hilden, Germany). U6 and GAPDH (Sangon Biotech, Beijing, China) were used as internal controls for the relative quantification of miRNA and mRNA expression, respectively. The expressions of miRNAs (221 and 23b) and the mRNAs (Smad2, Smad7, fibronectin, TGF-β1, MMP9, col I, P62, LC3, Beclin, mTOR, and α-SMA) were quantified. The miRNA primers were designed using http://www.srnaprimerdb.com from the miRNA mature sequence that was deposited in the miRNA database https://www.mirbase.org/. The sequence of primers of studied genes is listed in table 1. The relative expression of both miRNA and mRNA was calculated in a final volume of 20 µL including 10 µL TOPreal SYBR Green (Cat. No. RT500S, Enzynomics, Korea), 1 µL each of forward and reverse primer (synthesized by Sangon Biotech, Beijing, China). The relative expression was calculated with the $2^{-\Delta\Delta ct}$ method. Briefly, Δct was calculated as the ct difference between the target gene and reference gene expression, and then ΔΔ ct was calculated as the difference between Δct of the sample and the average Δct of the control.

Histopathological Study

After paraffin block formation, 5 µm thick sections were stained by hematoxylin and eosin (H&E). The Mallory Trichrome (MT) stain (Bio-Optica,

Table 1: Primer sequence of the studied genes

Gene	Forward primer	Reverse primer	Size	Accession no.
TGF-β1	AGGGCTACCATGCCAACTTC	CCACGTAGTAGACGATGGGC	168	NM_021578.2
Smad-7	GAGTCTCGGAGGAAGAGGCT	CTGCTCGCATAAGCTGCTGG	84	NM_030858.2
Smad-2	CCGTCGGAAGAGGAAGGAAC	AAATCTACCCTGCACCCAGC	80	NM_019191.2
Beclin-1	GAATGGAGGGGTCTAAGGCG	CTTCCTCCTGGCTCTCTCT	180	NM_001034117.1
LC-3	GAAATGGTCACCCCACGAGT	ACACAGTTTTCCCATGCCCA	147	NM_012823.2
mTOR	GCAATGGGCACGAGTTTGTT	AGTGTGTTCACCAGGCCAAA	94	NM_019906.2
P62	GGAAGCTGAAACATGGGCAC	CCAAGGGTCCACCTGAACAA	183	NM_181550.2
Col-1	GCAATGCTGAATCGTCCCAC	CAGCACAGGCCCTCAAAAAC	176	NM_053304.1
Gapdh	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	143	NM_017008.4
Mmp9	GATCCCCAGAGCGTTACTCG	GTTGTGGAAACTCACACGCC	132	NM_031055.2
Fibronectin	GGATCCCCTCCCAGAGAAGT	GGGTGTGGAAGGGTAACCAG	188	NM_019143.2
α-SMA	ACCATCGGGAATGAACGCTT	CTGTCAGCAATGCCTGGGTA	191	NM_031004.2
U6	GCTCGCTTCGGCAGCACA	GAGGTATTCGCACCAGAGGA		
Mir-221	AACAAGGGGTTCCTGGCAT	GTCGTATCCAGTGCAGGGT		
Mir-23b	AACAAGGGGTTCCTGGCAT	GTCGTATCCAGTGCAGGGT		
	microRNA RT primers			
U6	AACGCTTCACGAATTTGCGTG			
Mir-221	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAATCA			
Mir-23b	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAATCA			

TGF-β1: Transforming growth factor-beta 1; *Smad-7: Mothers against decapentaplegic homolog-7*; *Smad-2: Mothers against decapentaplegic homolog-2*; *LC-3: Microtubule-associated protein 1A/1B-light chain 3*; *mTOR: Mechanistic Target of Rapamycin Kinase*; *P62: ubiquitin-binding protein p62*; *Col-1: collagen type 1*; *Gapdh: glyceraldehyde-3-phosphate dehydrogenase*; *Mmp9: Matrix metallopeptidase 9*; *α-SMA: alpha-smooth muscle actin*

Milano, Italy) was used for staining collagen fibers and estimating fibrosis. A Light microscope (LEICA ICC50 W, Leica, Wetzlar, Germany) was used to examine the slides. The Image J software (Version 1.53m, NIH, USA) was used to calculate the percentage of collagen.

Immunohistochemical Staining

Deparaffinization and rehydration of liver sections were followed by incubation at 3% hydrogen peroxide in PBS for 15 min. Incubation overnight with primary antibodies of Beclin1 (1:200, #3738, MA, USA), LC3A/B (1:500, #12741, Cell Signaling Technology), or SQSTM1/ P62 Rabbit mAb (1: 250, #23214, Cell Signaling Technology) was performed.[18] Then, the addition of a secondary antibody and avidin-biotin complex (Vectastain® ABC-peroxidase kit, Vector Laboratories, Burlingame, CA, USA) was performed. Diaminobenzidine (DAB) (Vector Laboratories, Inc., Burlingame, USA) was added to develop color. The Image J software (Version 1.53 m, NIH, USA) was used to estimate the positive area.

Statistical Analysis

The collected data were analyzed using GraphPad Prism 9 software (Version 9.0.1, Inc., Boston, USA). Continuous quantitative variables were expressed as mean±SEM and categorical qualitative variables were expressed as absolute number and relative frequencies (percentage). The Shapiro-Wilk test was used to test the normality of the data. Kruskal Wallis with a nonparametric *post hoc* test (Dunn's test) was used for pairwise comparisons. The results were considered statistically significant when the probability was less than 0.05 ($P<0.05$).

Results

Characterization and Homing of hUCB-MSCs and their Derived Exosomes.

The present study showed that the extracted cells were round on the 3rd day of culture (figures 1A and B). They appeared elongated, spindle, and fibroblastic on the 7th day of culture. Additionally, the isolated cells were positive for the MSCs surface markers: CD106, CD105, CD90, CD73, and CD44, and were negative for the hematopoietic stem cells markers: CD34, CD45, and CD11b (figures 1C-J). The homing of PKH-26 labeled hUCB-MSCs into the cirrhotic liver was confirmed by the presence of bright red dots (figure 1K).

However, the isolated MSCs-derived exosomes were positive for CD63 and CD9 and negative for CD81 (figures 2A-C). Moreover, the TEM examination illustrated that the hUCB-MSCs-derived exosomes displayed a heterogeneous population of small varying sizes vesicles (figure 2D). The fluorescent

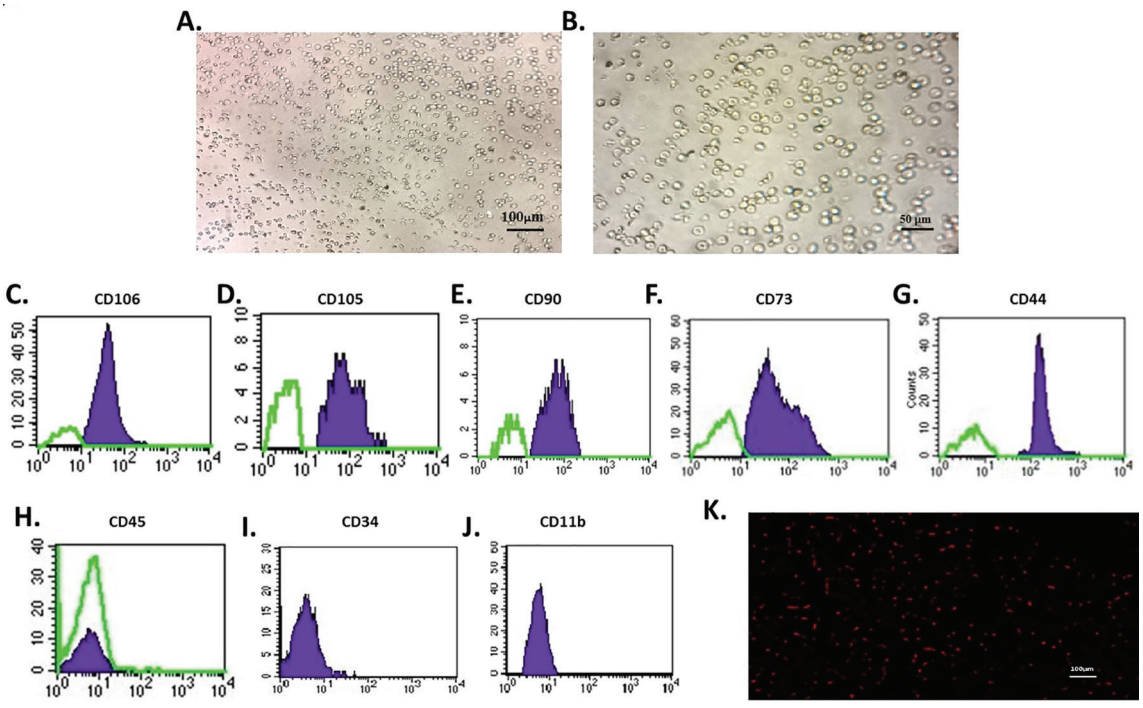

Figure 1: The isolation, identification, and homing of hUCB-MSCs. A and B: photomicrographs show human umbilical cord blood-mesenchymal stem cells (hUCB-MSCs) on the 3rd day of culture (×100 and ×200, respectively) C-G: Histograms show that hUCB-MSCs were positive for CD106, CD105, CD90, CD73, and CD44, respectively. H-J: Histograms show that hUCB-MSCs were negative for CD45, CD34, and CD11b, and K: A photomicrograph shows the homing of PKH-26 labeled hUCB-MSCs into the cirrhotic liver appearing as bright red dots.

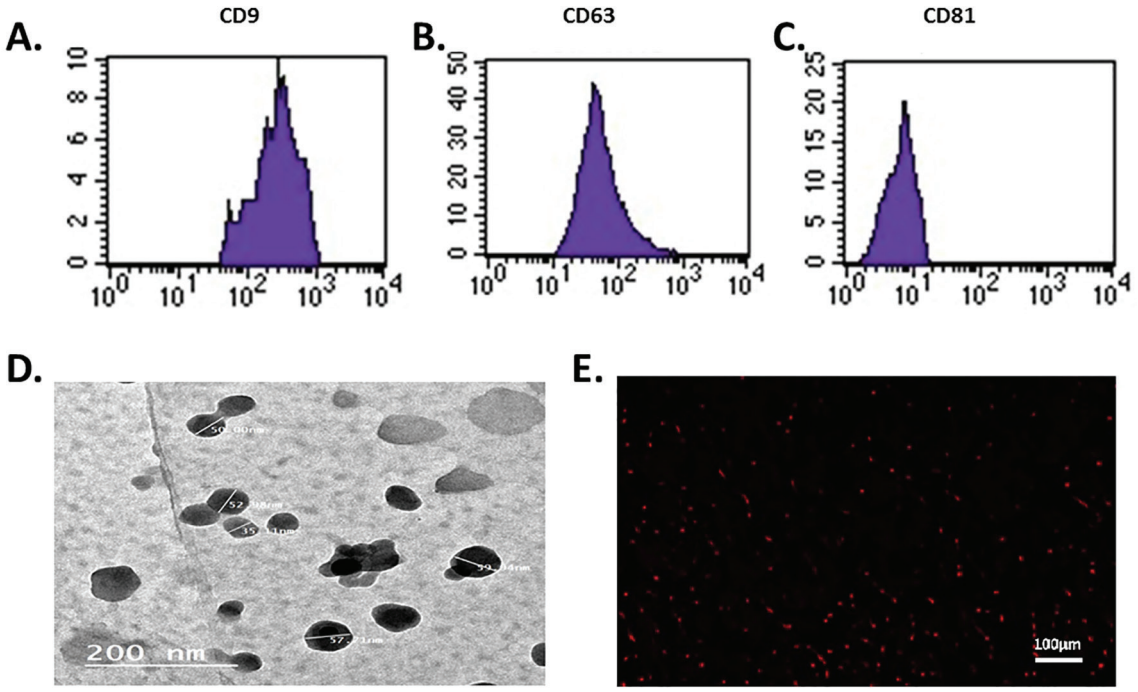

Figure 2: The characterization and homing of human umbilical cord blood-mesenchymal stem cells (hUCB-MSCs) derived exosomes. A-C: Histograms show that hUCB-MSCs derived exosomes were positive for CD9 and CD63, and were negative for CD81, D: Transmission electron microscopy imaging of hUCB-MSCs derived exosomes showing a heterogeneous population of small varying sizes vesicles, and E: A photomicrograph shows homing of PKH-26 labeled hUCB-MSCs-derived exosomes into the cirrhotic liver appearing as bright red dots.

microscopy examination of the deparaffinized unstained sections from the livers of MSCs-derived exosomes treated groups showed a red fluorescent emission confirming the homing of the PKH-26-stained exosomes to the cirrhotic livers (figure 2E).

Effect of Administration of hUCB-MSCs and/or their Exosomes on the Liver Function Tests

A significant elevation in ALT, AST, and ALP in addition to a significant decline of albumin were detected in the cirrhotic group compared with the control one (P<0.001). The treatment with hUCB-MSCs, exosomes, or the combination of them significantly reduced ALT, AST, and ALP and significantly elevated albumin in order compared to the cirrhotic group. No significant differences were found between the group that received both hUCB-MSCs and exosomes and the control group regarding ALT, ALP, or albumin (figure 3).

Effect of Administration of hUCB-MSCs and/or their Derived Exosomes on the Expression of miRNA and Autophagy and Fibrotic Pathways

Compared with the control rats, the cirrhotic rats showed a highly significant upregulation in the expression of *miRNA-221* (1.00±0.09 versus 4.20±0.51), *TGF-β1* (1.01±0.12 versus 3.98±0.78), *MMP9* (1.01±0.15 versus 6.59±0.79), *fibronectin* (1.02±0.19 versus 8.15±0.82), *collagen I* (1.01±0.17 versus 6.94±0.65), *α-SMA* (1.02±0.18 versus 7.68±0.66), *Smad2* (1.01±0.14 versus 12.93±0.85), and P62 (1.01±0.17 versus 6.61±0.91) (P<0.001, for each). In contrast, a significant downregulation was found in the cirrhotic group in the expression of *Smad7* (1.01±0.11 versus 0.29±0.03), *miRNA-23* (1.043±0.29 versus 0.56±0.10), *mTOR* (1.04±0.29 versus 6.26±0.30), *Beclin* (1.01±0.14 versus 0.28±0.09), and *LC-3* (1.00±0.01 versus 0.18±0.01) compared to the control group (P<0.001, for each) (figures 4 and 5).

The treatment of cirrhotic rats with conditioned media significantly downregulated *fibronectin* (4.93±0.19), *collagen I* (5.25±0.51), *α-SMA* (6.00±0.23), and *Smad2* (10.42±1.01) (figure 4).

The treatment of cirrhotic rats with hUCB-MSCs, exosomes, or the combination of them significantly downregulated *miRNA-221* (2.26±0.17, 1.59±0.09, 1.05±0.19), *MMP9* (2.54±0.17, 1.97±0.05, 1.27±0.04), *fibronectin* (2.94±0.32, 2.10±0.07, 1.76±0.18), *collagen I* (2.81±0.07, 2.28±0.10, 1.84±0.12), *α-SMA* (3.89±0.31, 3.46±0.25, 2.10±0.21), *Smad2* (4.96±0.19, 2.73±0.09, 2.05±0.4) (P<0.001, for each), and P62 (3.04±0.07, 2.26±0.15, 1.83±0.31) (P=0.032, P<0.001, P<0.001, respectively). Additionally, the treatment of cirrhotic rats with hUCB-MSCs,

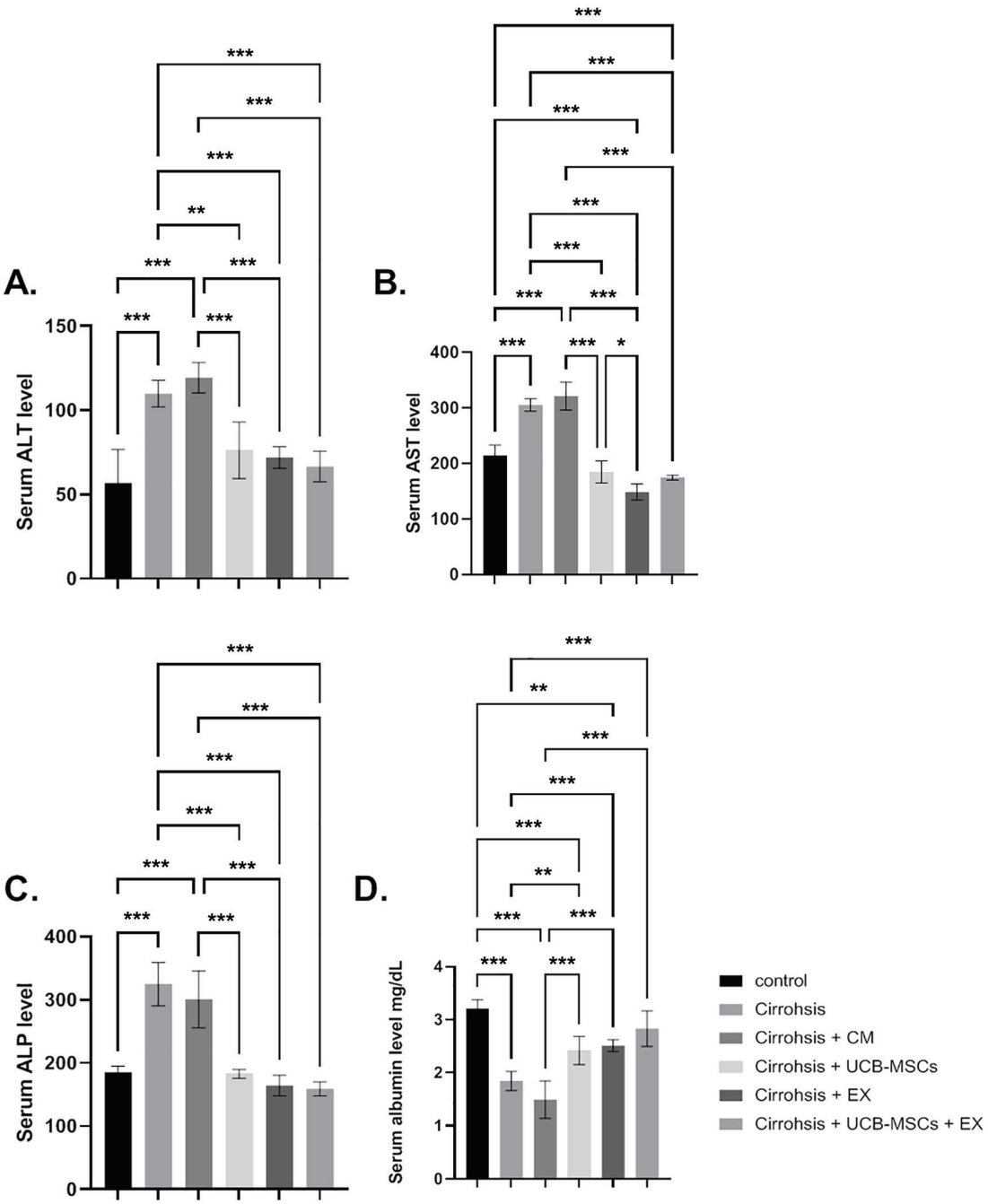

Figure 3: The effect of human umbilical cord blood-mesenchymal stem cells (hUCB-MSCs) and/or their derived exosomes on the liver function test. A: serum alanine transaminase (ALT) (U/L); B: serum aspartate transaminase (AST) (U/L); C: Alkaline phosphatase (ALP) (U/L); and D: serum albumin level (gm/dL). Values are the mean of nine rats per group±SEM; *P<0.05; **P<0.01; and **P<0.001

exosomes, or the combination of them significantly upregulated *mTOR* (3.42±0.35, 2.65±0.27,1.97±0.08), *Beclin* (0.81±0.08, 0.99±0.09,1.05±0.12),*LC3*(0.71±0.04,0.76±0.05,1.01±0.06) (P<0.001, for each), and *Smad7* (0.60±0.02,0.83±0.07,0.97±0.04),and*miRNA-23*(1.87±0.35, 2.54±0.35, 3.22±0.19) (P=0.021, P<0.001, P<0.001, respectively) (figures 4 and 5).

However, the downregulation of TGF-β1 was detected in the rats that received exosomes either alone (2.19±0.14), or in combination with hUCB-MSCs (1.85±0.35) (P=0.036 and P=0.013, respectively) (figure 4).

The best improvement in gene expression was detected in the group that received a combination of hUCB-MSCs and exosomes where no significant difference with the control group was detected in all genes except in *miRNA-23* (P<0.001). While in the group that received exosomes only, insignificant differences with the control group were seen in *α-SMA*, *miRNA-23*, and *LC3* (P<0.001 for each), and *mTOR* (P=0.024).

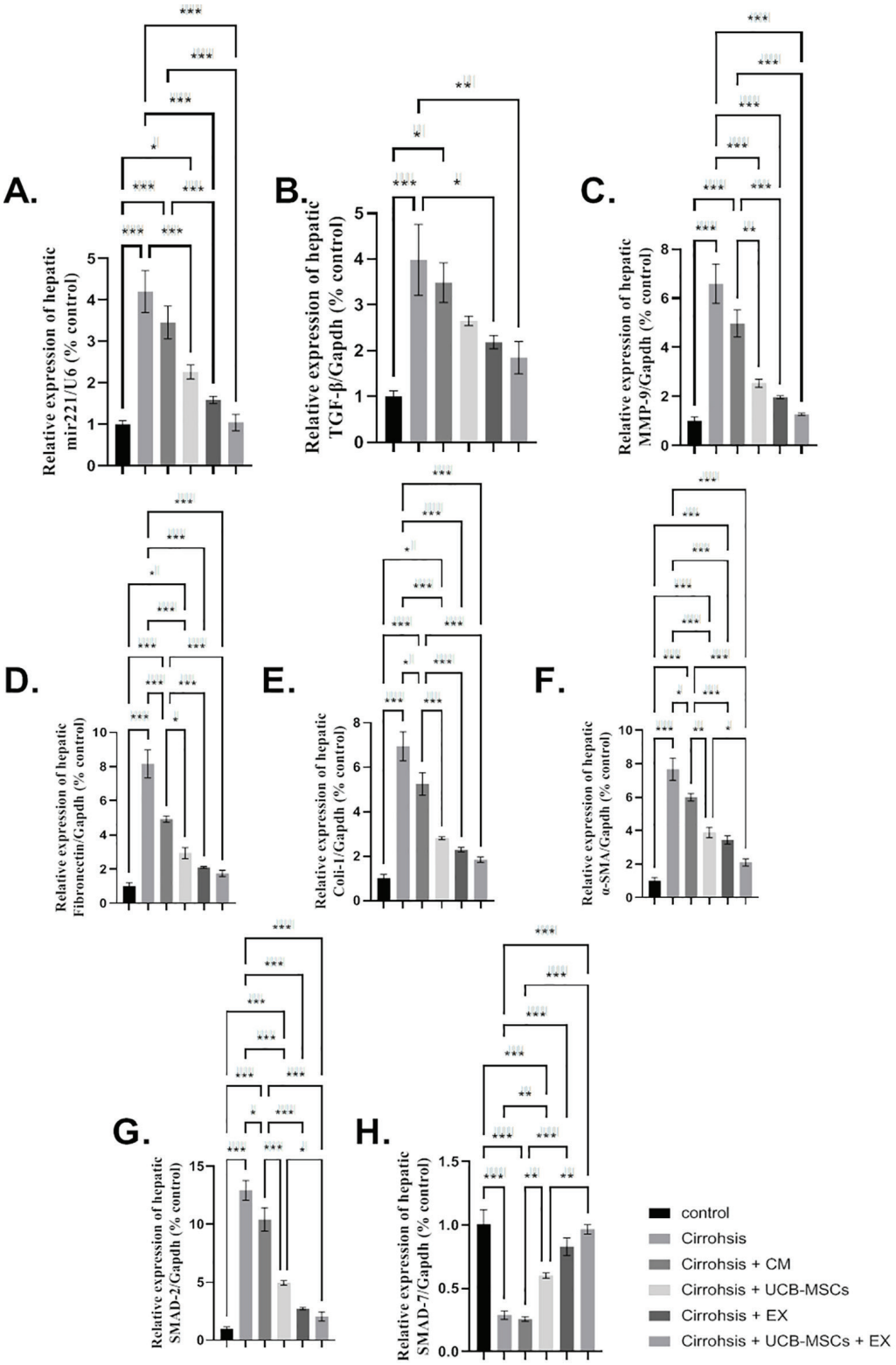

Figure 4: The effect of human umbilical cord blood-mesenchymal stem cells (hUCB-MSCs) and/or their derived exosomes on the expression of the hepatic fibrotic markers and miR-221 (A-G). A: relative expression of miR-221/U6; B: relative expression of *TGF-β1/Gapdh*; C: relative expression of *MMP-9/Gapdh*; D: *A/Gapdh*; G: relative expression of *Smad-2/Gapdh,* H: relative expression of *Smad-7/Gapdh.* Values are mean of nine rats per group±SEM; *P<0.05; **P<0.01; and ***P<0.001

In the cirrhotic rats that received hUCB-MSCs, there was no significant difference compared to the control group in the gene expression of *TGF-β1*, *MMP9*, *miRNA-23*, and *Beclin*. In the cirrhotic rats that received conditioned media, there were significant differences with the control rats regarding all studied genes except in *miRNA-23* (figures 4 and 5).

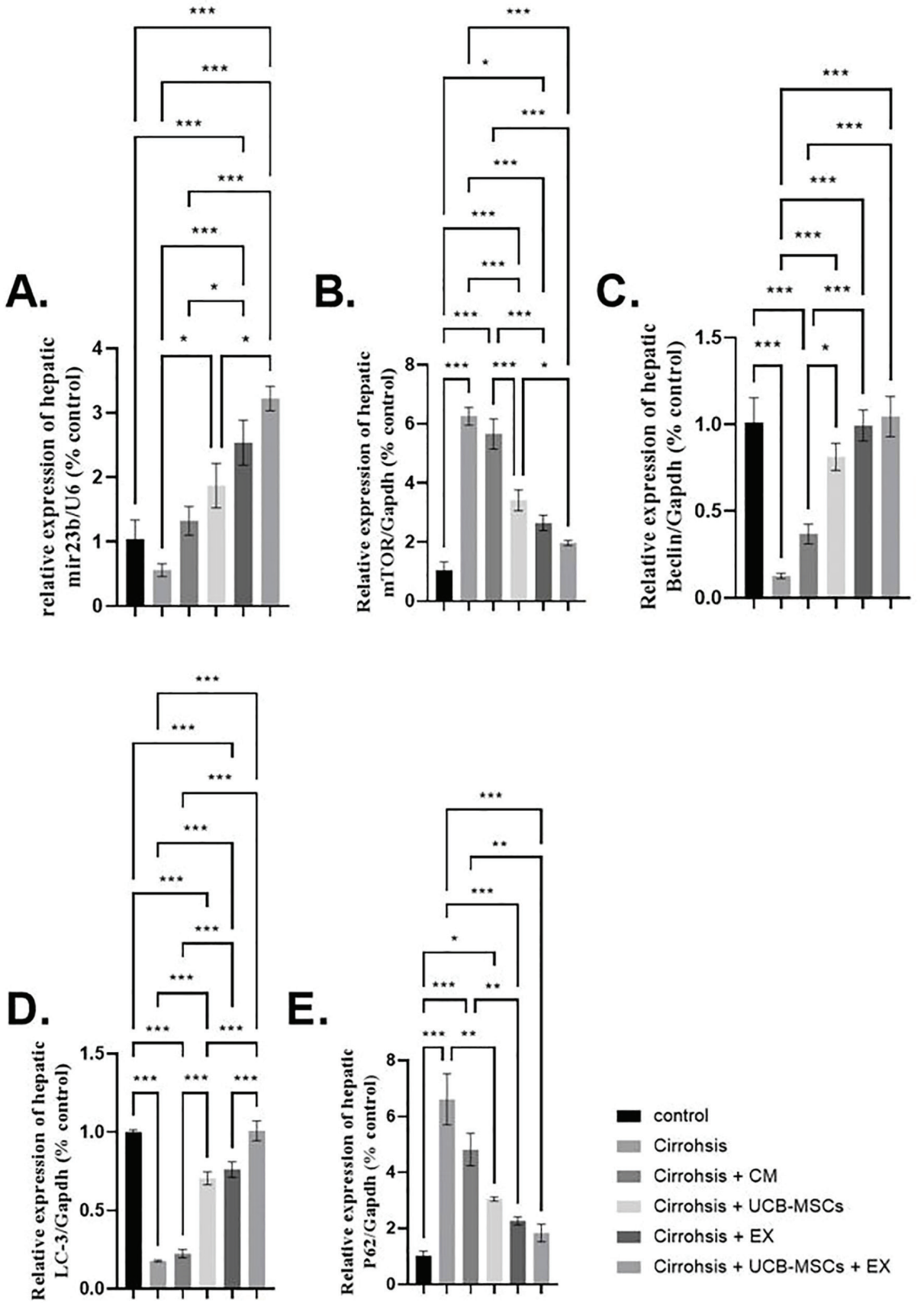

Figure 5: The effect of human umbilical cord blood-mesenchymal stem cells (hUCB-MSCs) and/or their derived exosomes on the expression of the hepatic autophagy markers and miR-23b (A-G); A: relative expression of miR-23b/U6; B: relative expression of *mTOR/Gapdh*; C: relative expression of *Beclin-1/Gapdh*; D: relative expression of *LC3-II/Gapdh*; and E: relative expression of *P-62/Gapdh*. Values are mean of nine rats per group±SEM; *P<0.05; **P<0.01; and ***P<0.001

Effect of Administration of hUCB-MSCs and/or their Derived Exosomes on the Histopathological Changes

Light microscopic examination of hepatic tissues from all experimental groups is illustrated in figure 6. The hepatic tissues from the control group revealed normal hepatic lobules and a normal construction of hepatocytes, with no appearance of any pathological changes (figure 6a). Whereas the cirrhotic group (figures 6 b and c) and the cirrhotic group that received conditioned media (figures 6 g and h) showed that most of the hepatocytes contained pyknotic nuclei and others had pyknotic nuclei and vacuolated cytoplasm.

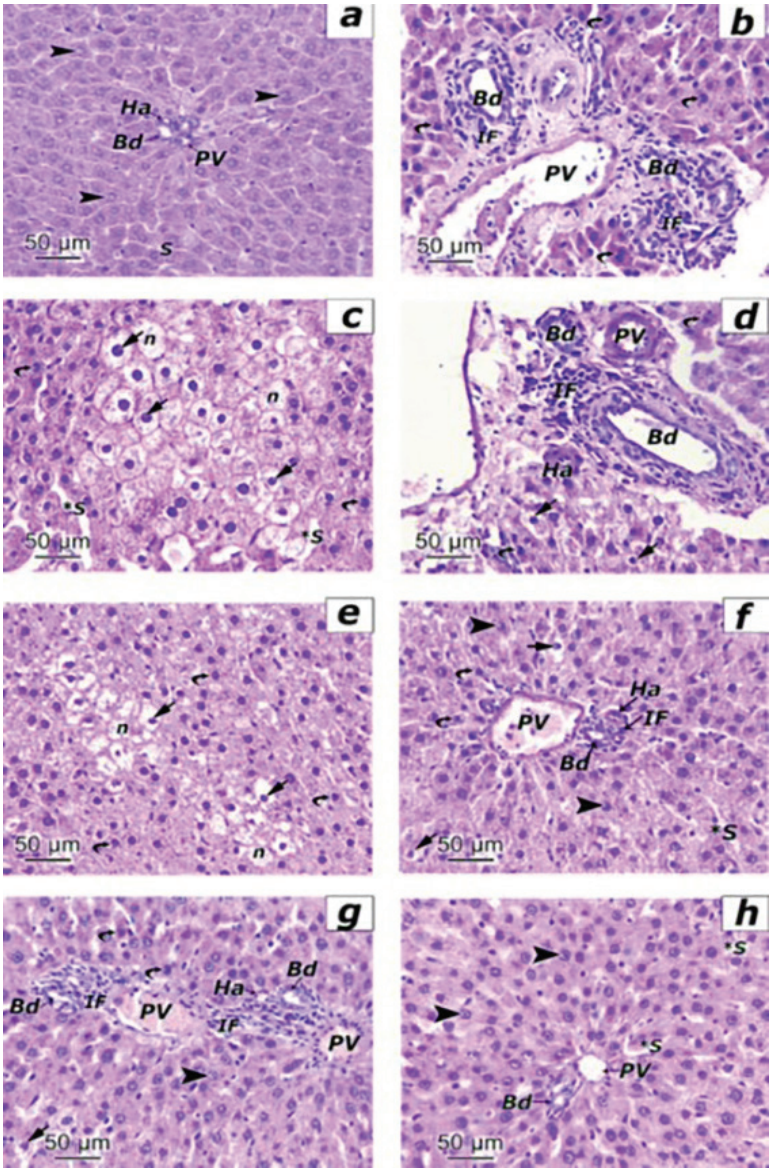

Figure 6: The effect of hUCB-MSCs and/or their derived exosomes on the H&E-stained rat hepatic tissues of different experimental groups (a-h). a: The control group shows the normal architecture of the liver with tightly packed cords of polygonal hepatocytes with rounded vesicular nuclei and acidophilic cytoplasm (arrowheads) radiating from the portal area containing the portal vein (PV), hepatic artery (Ha), bile duct (Bd) and separated by well-organized blood sinusoids (S). b and c: The cirrhotic group shows disrupted liver architecture around dilated, thickened, and irregular-walled portal vein (PV) that is surrounded by monocellular infiltrations (IF). Most hepatocytes demonstrate pyknotic nuclei (curved arrow) and others with pyknotic nuclei and vacuolated cytoplasm (short arrow) and separated by dilated blood sinusoids (S*). Foci of vacuolated hepatocytes (n) can be observed. d and e: The cirrhotic+conditioned media group displayed the same pathological changes as group 2, hepatocytes with pyknotic nuclei (curved arrow) and others with pyknotic nuclei and vacuolated cytoplasm (short arrow). Thick-walled bile duct and few monocellular infiltrations (IF) can be noticed. f: The cirrhotic+hUCB-MSCs group shows a partially normal appearance of hepatocytes with the slightly dilated portal vein (PV), bile duct duplication (Bd), hepatic artery (Ha), and monocellular infiltrations (IF). Moreover, some hepatocytes still exhibited pyknotic nuclei (curved arrow) and vacuolated cytoplasm (short arrow). g: The cirrhotic+exosomes group shows a nearly normal appearance of hepatocytes with a slightly dilated portal vein (PV). Most hepatocytes with rounded vesicular nuclei and acidophilic cytoplasm (arrowhead) and separated by slightly dilated blood sinusoids (S*). A few scattered hepatic cells with pyknotic nuclei (curved arrow) and vacuolated cytoplasm (short arrow) can be observed. Few mono-cellular infiltrations (IF) can be noticed. h: The cirrhotic+exosomes+hUCB-MSCs group shows a normal hepatic structure with the portal vein (PV) and the bile duct (Bd) surrounded by normal hepatocytes (arrowhead) and separated by well-organized blood sinusoids (S). Scale bar 50 µm, ×400.

Additionally, monocellular infiltrations particularly around the portal triad and necrotic foci of vacuolated hepatocytes can be observed. Dilated and thickened wall portal vein with bile duct duplication can be noticed. On the contrary, the cirrhotic group that received hUCB-MSCs retained a partially normal appearance of hepatic lobules, but some hepatocytes still displayed pyknotic nuclei and less vacuolated cytoplasm, and some mono cellular infiltrations can be noticed around the portal triad (figure 6d). The cirrhotic group that received exosomes retained the nearly normal appearance of hepatocytes where most hepatocytes have vesicular nuclei and acidophilic cytoplasm. Few mono-cellular infiltrations particularly around the portal triad can be noticed (figure 6e). The group that received both MSCs and exosomes showed a similar pattern to the control group (figure 6f).

Effect of Administration of hUCB-MSCs and/or their Derived Exosomes on the Collagen Disposition

Mallory Trichrome (MT)-stained hepatic tissues from the studied groups were illustrated in (figure 7). Hepatic tissues of control rats exhibited normal distribution of collagen fibers as fine fibers around the portal triad. Cirrhotic hepatic tissues displayed abundant collagen fiber distribution around the portal triad as well as in between the hepatocytes. In contrast, hUCB-MSCs and exosome recipient cirrhotic rats showed a decline in the concentration and arrangement of the collagen fibers surrounding the portal triad to a lesser degree. Interestingly, concomitant administration of MSCs and exosomes in cirrhotic rats exhibited scanty collagen fiber distribution around the portal triad similar to the control group. A morphometrical and statistical analysis of the area percentage of the distribution of collagen fibers showed a significant increase in the cirrhotic rats compared to the control rats. However, a significant reduction of the area percentage of collagen fibers was observed in MSCs and exosome groups and to a higher degree in the combination group.

Effect of Administration of hUCB-MSCs and/or their Derived Exosomes on the Beclin-1 Immunohistochemical Expression

Cirrhotic tissues exhibited a decrease in Beclin-1 expression shown as a significant decrease in the percentage of positive expression to the total area compared with the control group. However, the expression started to increase when the cirrhotic rats were given either hUCB-MSCS or exosomes with a significant increase in the percentage of positive immune-expression area compared with the cirrhotic tissue. Interestingly, combined treatment with hUCB-MSCS and exosomes brought back Beclin-1 expression to the level of the control group (figure 8).

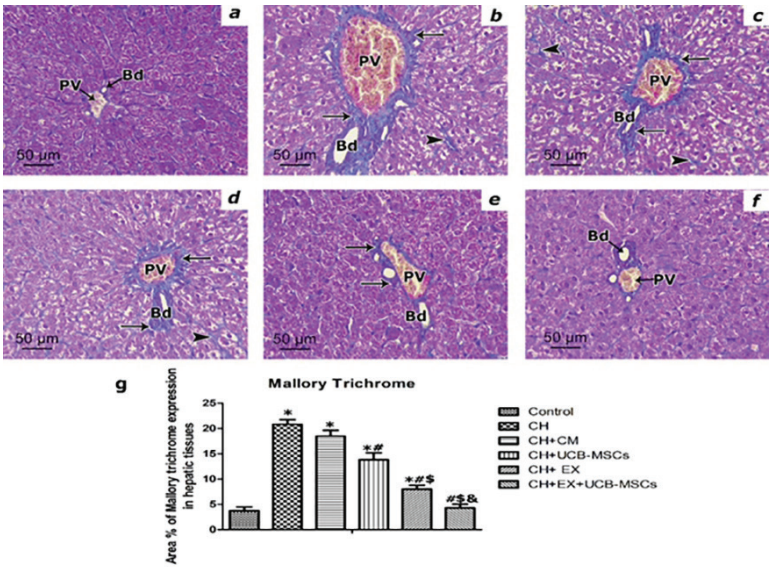

Figure 7: The Effect of human umbilical cord blood-mesenchymal stem cells (hUCB-MSCs) and/or their derived exosomes on the collagen deposition in Mallory's trichrome-stained hepatic tissues in different groups (a-F). a: control group, b: cirrhotic group, c: cirrhotic+conditioned media group, d: cirrhotic+hUCB-MSCs group, e: cirrhotic+exosomes group, and f: cirrhotic+exosomes+hUCB-MSCs group. Arrows and arrowheads refer to the blue staining of collagen fibers around the portal triad and in between the hepatocytes respectively that indicate fibrosis. (PV) refers to the portal vein (PV) and (Bd) refers to the bile duct. The bar chart shows the statistical analysis of the area % of collagen fiber distribution among the different groups. Data shown are the mean of nine rats per group±SEM. P<0.05 is considered significant. *Compared with the control group; #Compared with the cirrhotic group; $Compared to the cirrhotic+hUCB-MSCs group; &Compared to the cirrhotic+exosomes. Scale bar 50 µm, ×400

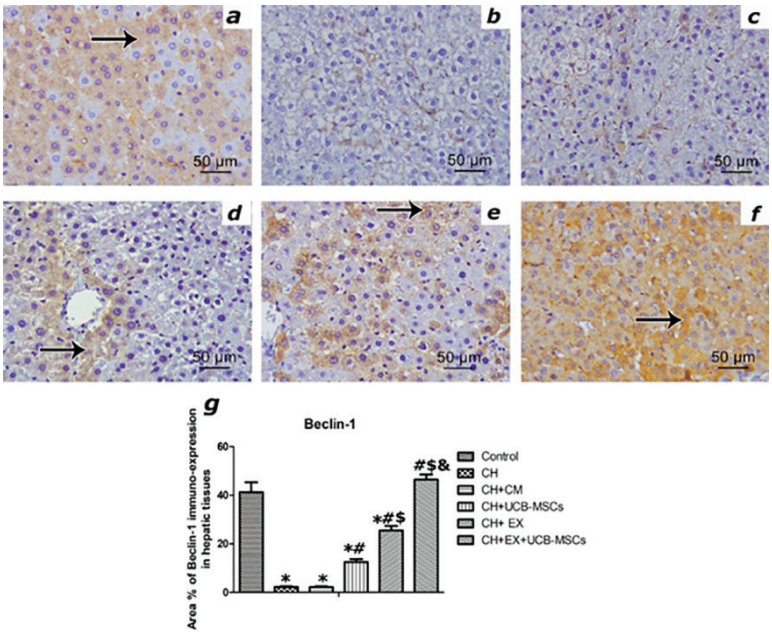

Figure 8: The effect of human umbilical cord blood-mesenchymal stem cells (hUCB-MSCs) and/or their derived exosomes on the immunohistochemical expression of autophagy-associated protein Beclin-1 among the different groups (a-f). a: control group, b: cirrhotic group, c: cirrhotic+conditioned media group, d: cirrhotic+hUCB-MSCs group, e: cirrhotic+exosomes group, and f: cirrhotic+exosomes+hUCB-MSCs group. The arrow refers to the brown staining of the cytoplasm of the hepatocytes, which indicates the positive immuno-expression of Beclin-1. The bar chart represents the statistical analysis of the area % of Beclin-1 immuno-positive expression among the different studied groups. Data shown are the mean of nine rats per group±SEM. P<0.05 is considered significant. *Compared with the control group; #Compared with the cirrhotic group; $Compared to the cirrhotic+hUCB-MSCs group; &Compared to the cirrhotic+exosomes. Scale bar 50 µm, ×400

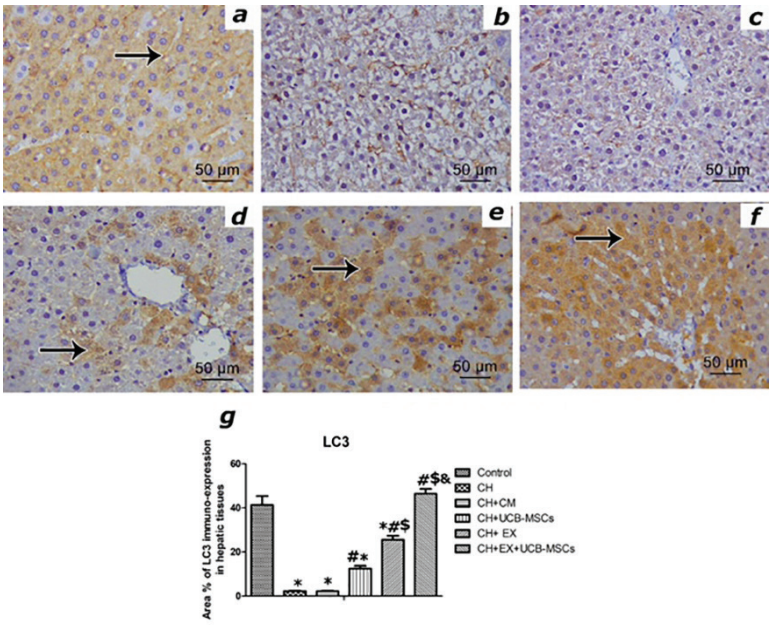

Figure 9: The effect of human umbilical cord blood-mesenchymal stem cells (hUCB-MSCs) and/or their derived exosomes on the immunohistochemical expression of autophagy-associated protein LC3 among the different groups. a: control group, b: cirrhotic group, c: cirrhotic+conditioned media group, d: cirrhotic+hUCB-MSCs group, e: cirrhotic+exosomes group, and f: cirrhotic+exosomes+hUCB-MSCs group. The arrows refer to the brown staining of the cytoplasm of the hepatocytes that indicates the positive immuno-expression of LC3. The bar chart represents the statistical analysis of the area % of LC3 immuno-positive expression among the different studied groups. Data shown are the mean of nine rats per group±SEM. P<0.05 is considered significant. *Compared with the control group; #Compared with the cirrhotic group; $Compared to the cirrhotic+hUCB-MSCs group; &Compared to the cirrhotic+exosomes. Scale bar 50 µm, ×400

Effect of Administration of hUCB-MSCs and/or their Derived Exosomes on the LC3-II Immunohistochemical Expression

Lower levels of LC3 were perceived in the cirrhotic group by a decrease in LC3 immune-positive cells. Following the treatment with exosomes, no further decrease in LC3 expression but showed relatively increased LC3

expression compared to the hUCB-MSCS group. Concomitant administration of hUCB-MSCS with exosomes in the cirrhotic rats led to an upregulation of LC3 levels that were comparable to that of the hUCB-MSCS or exosomes alone (figure 9).

Effect of Administration of hUCB-MSCs and/or their Derived Exosomes on the P62 Immunohistochemical Expression

P62 immunostaining revealed positive expression in the control group. Increased P62 expression was noted in cirrhotic livers shown as a significant increase in the percentage of immune-positive areas. Following the administration of hUMB-MSCs and exosomes separately, the P62 immuno-expression was downregulated compared to the control representing a significant decline in the percentage of immune-positive areas compared to cirrhotic livers, but they still exhibited a significant difference from the control group. However, concurrent administration of both hUCB-MSCS and exosomes showed a decrease in P62 expression compared to the cirrhotic liver. This effect was comparable to the control group (figure 10).

Discussion

In our study, treatment with hUCB-MSCs enhanced liver function in cirrhotic rats, as evidenced by a decrease in ALT, AST, and ALP levels and an increase in albumin levels. Similar to our results, MSC transplantation slowed the progression of liver cirrhosis in both human and animal models.[19] Moreover, hUCB-MSCs ameliorated liver function in patients with HBV-related cirrhosis in the clinical trial performed by Shi and colleagues.[20] Patients had a higher survival rate with marked improvement in serum albumin, prothrombin time, cholinesterase, and total bilirubin. Furthermore, autologous BM-MSCs-derived hepatocyte-like cell transplantation showed a therapeutic effect in patients with end-stage liver failure. This improvement was demonstrated by serum albumin level, Model for End-Stage Liver Disease (MELD) score (calculated using serum bilirubin, serum creatinine, and International Normalized Ratio (INR), tiredness scale, and performance status.[21]

In our study, the administration of hUCB-MSCs significantly downregulated *miRNA-221*, *MMP9*, *fibronectin*, *collagen I*, *α-SMA*, *Smad2*, and P62. Additionally, it significantly upregulated *mTOR, Beclin, LC3, Smad7,* and *miRNA-23.*

Several mechanisms are involved in the amelioration of liver cirrhosis after the administration of MSCs. MSCs home into the damaged liver and differentiate into hepatocyte-like cells *in vivo*, enhance hepatocyte proliferation

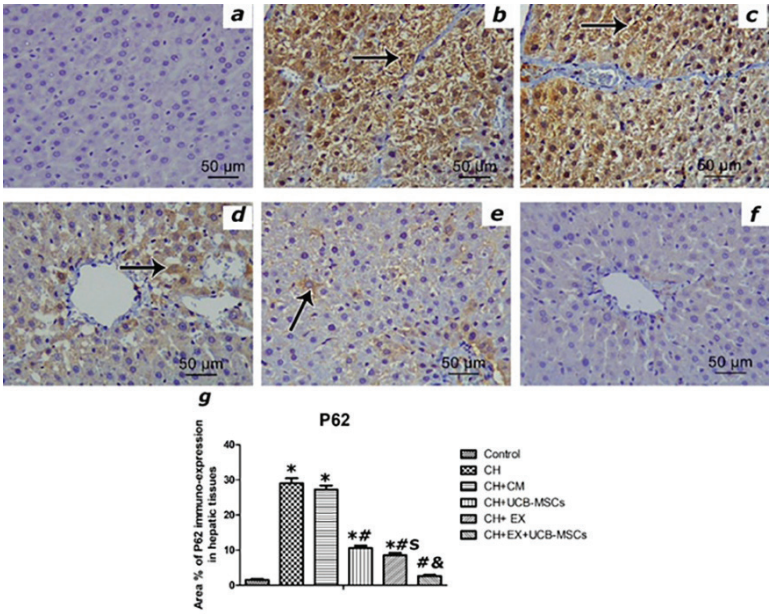

Figure 10: The effect of hUCB-MSCs and/or their derived exosomes on the immunohistochemical expression of autophagy-associated protein P62 among the different groups. a: control group, b: cirrhotic group, c: cirrhotic+conditioned media group, d: cirrhotic+hUCB-MSCs group, e: cirrhotic+exosomes group, and f: cirrhotic+exosomes+hUCB-MSCs group. The arrows refer to the brown staining of the cytoplasm of the hepatocytes, which indicates the positive immuno-expression of P62. The bar chart represents the statistical analysis of the area % of P62 immuno-positive expression among the different studied groups. Data shown are averages with SE from nine rats in each group. P<0.05 is considered significant. *Compared with the control group; #Compared with the cirrhotic group; $Compared to the cirrhotic+hUCB-MSCs group; &Compared to the cirrhotic+exosomes. Scale bar 50 µm, ×400.

by producing high levels of the hepatic growth factor, and have an immunomodulatory role in decreasing inflammation by the production of inhibitory cytokines or stimulating the development of regulatory T cells. Furthermore, MSCs can decrease HSC proliferation and α-SMA expression in a Notch-dependent manner. Besides, MSCs caused a significant decline in collagen expression with attenuated fibrosis. Additionally, MSCs caused greater breakdown of collagen and other ECM proteins in the cirrhotic liver. Moreover, TGF-β is responsible for the suppressing role of MSCs on T-cell proliferation as well as their paracrine inhibitory effect on cytotoxic T lymphocytes and natural killer cells.[22] On the other hand, the administration of MSCs has some drawbacks including the need for a regular cell supply with a stable phenotype, high expenses, time needed for the isolation and processing, possible ectopic tissue development, and cell trapping in the pulmonary vessels.[23]

In our study, the treatment of cirrhotic rats with conditioned media significantly downregulated the hepatic fibrosis markers (fibronectin, collagen I, α-SMA, and Smad2). However, improvement in liver functions was limited only to ALT and albumin. Similar to our findings, Fiore and colleagues and Huang and colleagues found that MSC-conditioned medium (MSC-CM) showed some improved results,[19, 24] implying that the possible therapeutic effect of MSCs *in vivo* is via their secretomes. Phinney and colleagues found that MSC-CM, by their secreted factors, microvesicles, and exosomes, had a therapeutic effect equivalent to that of MSCs in acute liver failure.[24, 25] Additionally, MSC-CM inhibited fibrosis by reducing the infiltration of pro-fibrogenic TGF-β-producing macrophages and by enhancing the apoptosis of HSCs.[26] Moreover, reduced expression of pro-inflammatory and pro-fibrotic markers (such as tumor necrosis factor- α [TNF-α], TGF-β, α-SMA, and col1) was observed in the livers of mice with non-alcoholic steatohepatitis that received MSC-CM derived from human exfoliated deciduous teeth.[27] The MSC-CM therapy resulted in a decreased percentage of apoptotic cells as well.[28]

The possible therapeutic effect of MSC-CM is *via* MSC-secreted extracellular vesicles including exosomes. Because of the merits of MSC-derived extracellular vesicles, such as microvesicles and exosomes, a new cell-free approach may be an alternate.[29, 30] They are smaller, simpler, easier to produce and store, and free of viable cells, so there is no risk of tumor development. Furthermore, they have lower immunogenicity than their parent cells due to their lower membrane protein amount.[7]

In our study, the exosomes of hUCB-MSCs ameliorated liver cirrhosis by significantly downregulating pro-inflammatory and pro-fibrotic mediators (miRNA-221, MMP9, fibronectin, collagen I, α-SMA, Smad2). Additionally, they significantly upregulated hepatic autophagy markers (mTOR, Beclin, LC3, and miRNA-23). Similar to our findings, exosomes produced by miR-181-5p-overexpressing adipose-derived-MSCs significantly inhibited collagen I, vimentin, α-SMA, and fibronectin expression.[31] Additionally, other previous studies reported the beneficial effects of the exosomes derived from bone marrow (BM)-MSCs in cirrhotic liver models, where Zhang and colleagues attributed that ameliorative effect to the downregulation of pyroptosis-related proteins (NLRP3, caspase-1, GSDMD, cleaved caspase-1 and IL-1β), which were significantly upregulated in cirrhotic livers.[15] Additionally, Damania and colleagues attributed that effect to reduced oxidative stress.[10] Moreover, Rong and colleagues attributed it to inhibiting HSC activation through the Wnt/β-catenin pathway.[11] Furthermore, Tian and colleagues stated that hUCB-MSCs-derived exosomes induced proinflammatory macrophages into an anti-inflammatory phenotype. miR-148a was identified as the therapeutic effector of the exosomes by regulating the STAT3 signaling pathway.[32]

It is believed that the therapeutic efficacy of MSCs is attributed to their secreted extracellular vesicles by regulating cell-cell communication and transporting paracrine mediators in angiogenesis, tissue regeneration, and immune modulation.[33-35] Exosomes are the perfect carriers for protecting and delivering molecules to their targets. They protect enzymes or RNAs against destruction by enveloping them in their membranes and promoting their uptake by cellular endocytosis of exosomes. Additionally, tissue injury enhances exosome uptake by the microenvironmental acidity. Moreover, their tiny size promotes their transport through blood and other body fluids. MSC-derived exosomes can thus use paracrine and endocrine signals to facilitate cell communication in both nearby and remote locations.[36]

Concerning the role of autophagy in cirrhosis, our study showed significant downregulation in Beclin-1 and LC-3 mRNA and protein expression in the cirrhotic group compared with the control group. The administration of hUCB-MSCs, exosomes, or a combination of them significantly upregulated *Beclin-1* and *LC-3*. However, the treatment with conditioned media

showed no improvement in the expression of autophagy markers. Similar to our results, Park and colleagues found that the anti-fibrotic effect of tonsil-derived MSCs was attributed to upregulation of autophagy-related proteins (LC3, beclin-1, and ubiquitin) and reduction of TGF-β expression. Besides, modulation of autophagy was important for the differentiation of tonsil-derived MSCs into hepatocytes.[37]

In our study, the administration of hUCB-MSCs, exosomes, or a combination of them significantly downregulated the marker of autophagic degraded substrates (P62). Similar to our finding, P62 was downregulated after MSC administration by IHC staining of liver tissue and western blot in acute-on-chronic liver failure.[38]

MSCs' ability to modulate the autophagy of injured cells is responsible for organ repair. MSCs regulate autophagy in immune cells, responsible for injury-induced inflammation, attenuating their survival and proliferation with the fading of inflammation. Furthermore, MSCs can regulate autophagy in endogenous adult or progenitor cells, promoting the survival and differentiation of these cells with the regaining of functional tissue.[39]

From the above findings, the exosomes derived from hUCB-MSCs have a potential therapeutic effect on liver cirrhosis, which is better when combined with their originating cells. However, our findings are limited to the use of hUCB-MSCs and their derived exosomes. Other sources of stem cells and other sources of exosomes need further clarification. Moreover, the potential therapeutic effect of hUCB-MSCs and their derived exosomes was studied within the scope of autophagic and fibrotic markers. Further research is recommended to explore other possible mechanisms explaining this therapeutic effect.

Conclusion

The therapeutic effect of hUCB-MSCs in the treatment of liver cirrhosis is possibly mediated by their derived exosomes. The therapeutic effect of exosomes is demonstrated by improvement in the biochemical, molecular, histopathological, and immunohistochemical profiles. Possible mechanisms include the anti-fibrotic and anti-inflammatory effects besides modulation of autophagy. This is possibly mediated by regulating the expression of miR-23b, miR-221, and their downstream effectors.

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Conflict of Interest: None declared.

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