Research Square

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Effect of lyophilized exosomes derived from umbilical cord stem cells on chronic anterior cruciate ligament cell injury

Hon-Lok Lo
Department of Orthopedics, Kaohsiung Medical University Hospital
Sung-Yen Lin
Department of Orthopedics, School of Post-Baccalaureate Medicine, College of Medicine, Kaohsiung Medical University
Cheng-Jung Ho
Department of Orthopedics, Kaohsiung Medical University Hospital
Po-Yu Liu
College of Medicine, Kaohsiung Medical University
Shih-Hao Huang
Department of Orthopedics, Kaohsiung Medical University Hospital
Yeh Ming-kung
School of Pharmacy, Institute of Life Sciences, National Defense Medical Center
Shun Cheng Wu
Orthopaedic Research Center, Kaohsiung Medical University
Cheng-Chang Lu (cclu0880330@gmail.com)
Department of Orthopedics, School of Medicine, College of Medicine, Kaohsiung Medical University

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Abstract

Background:

Facilitating the healing process of injured anterior cruciate ligament (ACL) tissue is crucial for patients to safely return to sports. Stem cell derived exosomes have shown positive effects on enhancing the regeneration of injured tendons/ligaments. However, clinical application of exosomes in terms of storage and pre-assembly is challenging. We hypothesized that lyophilized exosomes derived from human umbilical cord stem cells (hUSC-EXs) could enhance the cell activity of chronically injured ACL cells.

Materials and Methods:

We harvested the 8 weeks injured ACL cells from rabbit under IACUC (No.110232) approval. The studied exosomes were purified from the culture medium of human umbilical cord stem cells (IRB approval No. A202205014), lyophilized to store, and hydrated for use. We compared exosome treated cells with non-exosome treated cells (control group) from the same rabbits. We examined the cell viability, proliferation, migration capability and gene expression of type I and III collagen, *TGFβ*, *VEGF*, and tenogenesis in the 8 weeks injured ACL cells after hUSC-EX treatment.

Results:

After hydration, the average size of hUSC-EXs was 85.2 nm, and the cells tested positive for the Alix, TSG101, CD9, CD63, and CD81 proteins but negative for the α-Tubulin protein. Compared with no exosome treatment, hUSC-EX treatment significantly improved the cell viability, proliferation and migration capability of 8 weeks injured ACL cells. In addition, the expression of collagen synthesis, *TGFβ*, *VEGF*, and tenogenesis gene were all significantly increased in the 8 weeks injured ACL cells after hUSC-EX delivery.

Discussion:

Lyophilized exosomes are easily stored and readily usable after hydration, thereby preserving their characteristic properties. Treatment with lyophilized hUSC-EXs improved the activity and gene expression of 8 weeks injured ACL cells.

Conclusion:

Lyophilized hUSC-EXs preserve the characteristics of exosomes and can improve chronically injured(8 weeks) ACL cells. Lyophilized hUSC-EXs could serve as effective and safe biomaterials that are ready to

use at room temperature to enhance cell activity in patients with partial ACL tears and after remnant preservation ACL reconstruction.

1. Background

Anterior cruciate ligament (ACL) injury is the most common sports injury around the knee joint.[1–3] Partial tears in the ACL occur in 10–28% of all ACL injuries.[4, 5] Two major concerns for the treatment of partial ACL tears are the extent of preserved knee stability and the quality of the injured ACL tissue, as these factors play pivotal roles in determining whether patients should undergo conservative treatment or opt for surgical reconstruction. ACL reconstruction with auto or allografts is recommended for individuals who have experienced a complete tear of the ACL, especially when there is concomitant injury to other knee ligaments or when conservative treatment has proven unsuccessful. After undergoing ACL reconstruction, the implanted graft undergoes a graft maturation process that involves stages such as graft necrosis, revascularization, recellularization, and remodeling.[6, 7] If the implanted graft does not mature promptly, it may sustain microtears, leading to retear or graft loosening. Several remnant preservation techniques have been proposed to enhance graft maturation after ACL reconstruction.[8–10] However, the quality and healing capability of injured ACL tissue, particularly during chronic injury, remain questionable. [11] Increased activity in chronically injured ACL cells is crucial for healing partial ACL tears and improving remnant tissue during ACL reconstruction.

Biologics such as growth factor, PRP, stem cell, and bio-scaffolds, have been proposed to enhance the regeneration of injured ACL cells in partial tears and after remnant preservation ACL reconstruction.[12] Exosomes, ranging in size from 50–200 nm, contain mRNAs, proteins and lipids and are secreted for intercellular comminution.[13, 14] Exosome treatment, as a “cell-free” therapy, offers advantages such as avoiding the risk of uncontrolled differentiation or reaction of implanted cells, the ability to target specific tissues, a higher safety profile, and lower immunogenicity.[15] Recently, stem cell derived exosome treatment has been applied to enhance the activity of injured tendon/ligament tissue.[16–18]

Among the various types of exosomes derived from various cell sources, human umbilical cord stem cell-derived exosomes (hUSC-EXs) have gained popularity in recent years.[19, 20] Umbilical cord stem cells possess high proliferation and differentiation capabilities, as well as the ability to prevent immune rejection, and involve less ethical and moral controversy.[21–23] The anti-inflammatory effects of hUSC-EXs have been demonstrated in osteoarthritic chondrocytes, and the ability of these cells to enhance the healing of injured tendons/ligaments has been demonstrated.[19, 20, 24] However, storing exosomes for clinical applications can be challenging, and an effective preservation method is crucial for allowing exosomes to be stored at room temperature while maintaining their biological capabilities and readiness to use. Presently, the suggested techniques for exosome storage primarily involve cryopreservation, spray drying, and freeze drying (lyophilization).[25] Lyophilized exosomes undergo dehydration and drying under low temperature and vacuum conditions, enabling storage and handling at room temperature while preserving their original activity.[25, 26]

In this study, we hypothesized that after hydration, lyophilized hUSC-EXs could maintain the biological characteristics of exosomes and enhance the cell activity of chronically injured ACL cells, subsequently improving the regeneration capability of ACL cells.

2. Materials and Methods

2.1 Harvesting of injured ACL tissue and cell culture

In this study, we aimed to investigate the effect of hUSC-EX treatment on chronically injured ACL cells. The injured ACL tissues were harvested from skeletally mature New Zealand male rabbits (n = 6) weighing 2.5–3.0 kg 8 weeks after ACL resection. The whole process was approved by the IACUC(KMU No.110232).

In brief, the rabbits were anesthetized via intramuscular injections of 40 mg/kg ketamine and 10 mg/kg xylocaine and maintained under gaseous anaesthesia of isoflurane (2%)/O_2.[27, 28] After the knee joint was exposed via a medial parapatellar approach, the ACL was detached from the femoral insertion.[11] The injured ACL tissues were harvested 8 weeks after the initial surgery. Subsequently, the ACL tissues were digested overnight using 5 mg/ml type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) in low glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) in a 5% CO2 incubator at 37℃\ and sub-cultured to obtain injured ACL cells.[29, 30] Passage three injured ACL cells were used for subsequent experiments.

2.2 Preparation and lyophilization exosomes derived from Umbilical Stem Cells

In the present study, the applied exosomes were derived from the culture medium of human umbilical stem cells. Human umbilical cord harvest, stem cell culture, exosome isolation, and lyophilization were conducted by "Precision Biotech Taiwan Corp" (product no. 1110817001).

2.2.1 Harvest of Human Umbilical Cords Stem Cells (hUSC)

Human umbilical cords were obtained from healthy mothers with full-term fetuses at Tri-Service General Hospital, Taiwan, with all donors providing informed consent before delivery. This study was approved by the Ethics Committee of Tri-Service General Hospital (approval no. A202205014). The method used for hUSC isolation and qualification were described in previous papers.[31, 32]

2.2.2 Prepare the lyophilized hUSC Exosomes (hUSC-EX)

The hUSC-EXs were obtained as described previously, with modificationsr.[33] Briefly, we cultured hUSCs in conditioned medium (Dulbecco's modified Eagle medium (DMEM) containing nutrient mixture F-12 (F-12; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 1% P/S, and 10 ng/mL basic fibroblast growth factor (bFGF; PeproTech, Cranbury, NJ, USA))[33], seeded

them in a Nunc™ Cell Factory™ System (Thermo Fisher) at a density of 3000 cells/cm^2, and incubated at 37℃ with humidified 5% CO2. When the cells reached 90% confluence, the culture suspension was collected for exosome isolation by serial centrifugation. The culture suspension was transferred to conical tubes for centrifugation at 300×g for 10 minutes at 4℃ to obtain the pellet. The supernatant was centrifuged at 2,000 ×g for 10 minutes at 4°C for the second time to remove cell debris, followed by centrifugation at 10,000 ×g for 30 minutes at 4°C for the third time to remove apoptotic bodies and other organelles. Finally, the supernatant was centrifuged at 120,000 ×g for 90 minutes at 4°C to obtain the exosome pellet.

The pellet was then resuspended in PBS and centrifuged again at 120,000 ×g for 90 minutes at 4°C for lyophilization preparation.[26] The lyophilization process was carried out as follows: the exosomes were prepared in distilled water and then frozen at -50°C to allow conversion to ice. Subsequently, over a period of 3 days, the pressure was reduced, and heat was applied to prevent sublimation of the frozen water in the material, resulting in a dry, structurally intact powder product. The final products were aseptically aliquoted into bottles and stored at 2°C ~ 30°C (Fig. 1).

2.2.3 Qualification of the lyophilized hUSC-EXs

The hUSC-EX powder was resuspended in PBS and subsequently quantified for size distribution and exosome markers.

2.2.3.1 Size distributions

Particle size measurements and data analysis were performed with a particle analyzer (qNano platform, iZON® Science) and Control Suite software v2.2 (iZON® Science), respectively, according to the manufacturer's protocol.

2.2.3.2 Exosome markers

We investigated the presence of exosomes positive for the markers Alix, TSG101, CD9, CD63, and CD81 and negative for the marker α-Tubulin.[34–37] The primary antibodies used in this study included CD9 (cat#60232-1-1AP), CD63(cat# 25682-1-AP), CD81 (cat# 66866-1-AP), Alix (cat# 12422-1-AP), TSG101 (cat #14497-1-AP), and α-Tubulin (cat# 66031-1-Ig) and were obtained from Proteintech (Chicago, IL, USA). The labeled proteins were visualized using a ChemiDoc™ XRS imaging system (Bio-Rad, Hercules, Cal, USA.). Western blotting was performed using a standard protocol.

2.3 Investigation of the change in cell activities of 8 weeks injured ACL cells after hUSC-EX uptake

2.3.1 Confirming the hUSC-EX uptake and internalization by injured ACL cells

hUSC-EXs were added to the 8 weeks injured ACL cells (10^{10} EVs particles/10^4 cells) culture medium for 6 h. The treated cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min. After washing

with PBS twice, 100 µL of 1X Phalloidin conjugate working solution (cat# ab176753; Abcam Cambridge, MA, USA) was added to stain the cytoskeleton of the treated cells. The cell slides were mounted, counterstained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) and observed via confocal microscopy (Olympus IX-81-FV100, Olympus, Tokyo, Japan).

2.3.2 Changes in cell activity (viability, proliferation, migration, and gene expression) after 8 weeks of injury in ACL cells after hUSC-EX uptake.

2.3.2.1 Cell viability

The cell viability of 8 weeks injured ACL cells was assessed in two groups: those treated with hUSC-EXs (n = 6) and those not treated with exosomes (control group; n = 6). The evaluation was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cat. No. M2003; Millipore Sigma).[29] We measured the 570 nm absorbance using a Bio-Rad Microplate Manager Benchmark Plus Reader (Bio-Rad Laboratories, USA).

2.3.2.2 Cell proliferation: EdU, Ki67 gene expression

The cell proliferation rate was measured with a Click-iT EdU (cell proliferation) assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and the expression of the *Ki67* gene, was assessed following the manufacturer's instructions. The cell slides were mounted and counterstained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA). The cell proliferation rate was calculated for five randomized areas per sample using ImageJ (64-bit Java v. 1.6.0_24; National Institutes of Health (NIH), Bethesda, Maryland, USA). The expression of the *Ki67* gene was calculated and is described in the RT-PCR section.

2.3.2.3 Transwell migration

The transwell migration assay was performed in a 6.5-mm Transwell™ chamber with an 8-µm pore diameter (Millipore EMD, Billerica, MA, USA). These cells were seeded at a density of 3×10^4 cells/per well in the upper chamber compartment with serum-free medium. DMEM supplemented with 10% (v/v) FBS was added to the lower chamber as a chemoattractant. After incubating at 37°C for 20 h, the cells were migrated to the lower membrane. The migrated cells were fixed and counted in each chamber under microscope observation. The relative migration rate was calculated as the ratio of the migration of each treatment group to that of the control group (% of the control).

2.3.2.4 Scratch assay

The hUSC-EX treated ACLs and cells not treated with exosomes were seeded at a density of 3×10^5 cells/per plate and incubated at 37°C with 5% CO2. When the cells reached > 90% confluence, the plate was scratched with a sterile 200-µL pipette tip. Cell migration status was observed under a microscope by closing the scratch gap at regular intervals (6 h, 12 h, 18 h, and 24 h). The relative changes in the cell migration rate were calculated with ImageJ (64-bit Java v. 1.6.0_24; National Institutes of Health (NIH), Bethesda, Maryland, USA).

2.3.3 Changes in immunofluorescence and gene expression:were evaluated for the following: Collagen I and III, VEGF, TGFB, tenogenic markers in 8 weeks injured ACL cells after hUSC-EX uptake.

We assessed the metabolic activity of injured ACL cells after hUSC-EX uptake by quantitatively measuring immunofluorescence and RT-PCR for Collagen Type I and III, *TGFβ*, *VEGF* and tenogenic markers (*Tenascin C* (*TNC*), and *Tenomodulin* (*TNMD*)).

2.3.3.1 Immunofluorescence

The hUSC-EX treated ACL cells and non-exosome treated cells were seeded at a density of 2×10^4 cells/well. After 24h of culture, the cells were fixed in 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min and blocked with 5% bovine serum albumin (BSA) in PBS. These cells were assessed for Collagen types I and III, TGFβ, and VEGF expression. These cells were stained with primary antibodies (all from Arigo Biolaboratories, Hsinchu, Taiwan; 1:200) overnight at 4°C, and then stained with the following fluorescent secondary antibodies: donkey anti-goat IgG (H + L)-FAM (Leadgene Biomedical, Tainan, Taiwan; 1:250) for Collagen types I and III and goat anti-mouse IgG (H + L)-TAMRA (Leadgene Biomedical; 1:250) for TGFβ and VEGF for 1h. The cells were subsequently rinsed thrice with PBS.[29] The slides were counterstained with DAPI (Thermo Fisher Scientific), mounted, and observed under a confocal microscope (Olympus FV-100, Olympus America, Inc)

2.3.3.2 RT-PCR

Total RNA from the ACL cells treated with hUSC-EXs (n = 6) and without exosomes (n = 6) was extracted using RNAzol reagent (Cat. No. RN-190; Molecular Research Center, USA), and 2 μg of total RNA was reverse-transcribed using the Maxima First Strand cDNA Synthesis Kit (Cat. No. K1642, Thermo Fisher Scientific) according to the manufacturer's instructions and described by Lu.[38] Real-time polymerase chain reaction (PCR) was carried out using SYBR Green PCR Master Mix (Cat. A25780, Thermo Fisher Scientific). The complementary DNA samples were amplified using primers according to Lu's study on ACL cells.[29] The cycling procedure was as follows: 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds. Threshold cycles (Ct) for each gene tested were normalized to the housekeeping gene GAPDH value (ΔCt), and every experimental sample was referred as its control (ΔΔCt). The experimental data of the hUSC-EX treated ACL cells are expressed as fold-changes ($2 - \Delta\Delta Ct$) compared to those of the nonexosome treated ACL cells, which were set as 1.

Statistical analysis

The differences between hUSC-EX treated ACL cells and non-exosome treated cells were analyzed using the *t*-test. All datas were presented as mean ± standard deviation (SD) with triple measurements. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using SPSS software version 20 (IBM, USA).

3. Results

3.1 Quantification of exosomes derived from human umbilical cord stem cells (hUSC-EXs)

The average size of hUSC-EXs was 85.2 nm, and the cells tested positive for the Alix, TSG101, CD9, CD63, and CD81 proteins, but negative for the α-Tubulin protein (Fig. 2). The results revealed that hydrated hUSC-EXs met the MISEV 2014 and 2018 criteria.[35–37]

3.2 hUSC-EXs were uptaken by the injured ACL cells

According to the IFC image, the delivered hUSC-EX (stained in red) was uptaken and internalized by the 8 weeks injured ACL cells (Fig. 3).

3.3 The 8 weeks injured ACL cells with increased viability, proliferation, and migration capability after hUSC-EX treatment.

After hUSC-EX treatment, the cell viability (Fig. 4A), the expression of EdU and *Ki67* gene (Fig. 4B), migration capability (transwell (Fig. 5A) and scratch migration (Fig. 5B)) was significantly higher in the hUSC-EX treated injured ACL cells compared to that of non-exosome treated group.

3.4 The hUSC-EX treatment significantly improved the gene expression of collagen synthesis, TGFB, VEGF and tenogenesis in 8 weeks injured ACL cells.

Moreover, compared with those in non-exosome treated cells, the gene expression of Collagen synthesis, *TGF-β*, *VEGF* (Fig. 6), and tenogenic markers (*TNC*, *TNMD*)(Fig. 7) of 8 weeks injured ACL cells were significantly improved after hUSC-EX treatment.

4. Discussion

Enhancing the activity of injured ACL cells is important for healing of partially injured ACL and remnant preserving ACL reconstruction. In this study, we examined the effect of lyophilized hUSC-EXs on chronically injured ACL cells. The average size of the hUSC-EXs was 85.2 nm, and the cells tested positive for the Alix, TSG101, CD9, CD63, CD81 protein, but negative for the α-Tubulin protein. These results met the MISEV 2014 and 2018 criteria for hUSC-EX quality. After hUSC-EX treatment, compared with non-exosome treated injured cells, 8 weeks injured ACL cells exhibited significantly improvements in cell viability, proliferation, migration capability and gene expression of collagen synthesis, *TGFβ*, *VEGF* and tenogenesis.

Stem cell derived exosomes have been proposed for the treatment of tendon/ligament injuries.[17, 18, 39] Wang et al.[17] used adipose stem cell (ASC) derived exosomes to treat chronic rotator cuff tears in rabbits. They found that the exosome treated group exhibited decreased fatty infiltration, higher histological scores, increased newly regenerated fibrocartilage, and improved mechanical properties. Lyu et al.[39] conducted a review on the use of ASC-derived exosomes for treating tendon injuries. Their

conclusion highlighted that ASC-derived exosomes promote tendon healing by reducing inflammatory responses, stimulating the proliferation and migration of tenocytes, promoting angiogenesis, and boosting collagen synthesis. Yu et al.[18] used bone marrow mesenchymal stem cell-derived exosomes to treat rat patellar tendon defects. The exosome-treated group exhibited increased histological scores, enhanced expression of collagen type I, mohawk, tenomodulin, and heightened proliferation of local tendon stem/progenitor cells *in vivo*. In our study, we observed that treatment with human umbilical cord stem cell derived exosomes (hUSC-EX) enhanced the cell viability, proliferation, migration capability and gene expression of collagen synthesis, *TGFβ*, *VEG*F, and tenogenesis in chronically injured ACL cells.

Exosomes from various sources exhibit distinct advantages and disadvantages.[40] While human umbilical cord mesenchymal stem cells may not be easily accessible, their advantages include a painless and noninvasive collection procedure, as well as a rapid self–renewal ability.[22, 23] Recently, exosomes derived from hUSC have increased in popularity for treating tendon/ligament injury.[19, 20] Han et al.[19] found that hUSC derived exosomes enhance the proliferation and migration capability of injured Achilles tendon cells *in vitro* and improve biomechanical properties. They also confirmed that this effect was mediated through the miR-27b-3p/ ARHGAP5/RhoA signaling pathway. In a study by Yao et al.[20], hUSC-derived exosomes were shown to enhance tendon-specific matrix components, improve biomechanical strength, and positively impact histological structure in a rat Achilles tendon injury model. These effects were attributed to the potential PTEN/mTOR/TGF-β1 pathway through the delivery of miR-29a-3p. Zhang et al.[41] found that hUSC exosomes could modulate macrophage polarization from M1 to M2 through the NF-κB signaling pathway, thereby improving the local inflammatory microenvironment. Taken together, our findings and those of others suggest that treatment with hUSC-derived exosomes could effectively enhance the activity of injured tendon/ligament cells *in vitro*, suggesting the potential to improve the healing of injured tissue *in vivo*.

The storage of exosomes is a major concern for clinical application. Ideally, the storage method should preserve the biological activities of exosomes, maintain structural stability, be convenient for transportation, be easily readable, and be clinically user friendly. The suggested technique for exosome storage primarily involves cryopreservation, spray-drying and freeze-drying (lyophilization).[25] The cryopreservation method results in the formation of “frostbite”, leading to the production of ice crystals inside the particles that can affect membrane stability. Storage at -80°C over time may alter the morphology and biological activity of the exosomes.[42, 43] Moreover, it is necessary to add antifreeze after cryopreservation to extend the storage life of exosomes.[44, 45] During the spray-drying technique, the atomization pressure and heating temperature also affect the stability of exosomes.[45] The lyophilization process involves complete dehydration and drying of the exosomes under low temperature and vacuum conditions, minimizing damage to biological structures.[25, 26] Additionally, lyophilized exosomes can be stored and maintained at room temperature and can be easily reconstituted by adding water.[26] In this study, we stored and rehydrated lyophilized hUSC-EXs at room temperature. The method preserved the size and expression of biological markers in exosomes and demonstrated the ability of the exosomes to effectively enhance the activity of injured ACL cells.

This study has several limitations. First, further investigations are needed to determine the optimal exosome dose and the possible mechanisms for enhancing injured ACL cells. Second, there was no comparison of the effects between fresh isolated exosomes and lyophilized exosomes on injured ACL cells. Third, we only investigated the exosome treated to 8 weeks injured ACL cells, the exosome enhancing effect to injured ACL cells more than 8 weeks is unknown. Finally, an animal study is needed to confirm the effectiveness of lyophilized hUSC-EXs for the treatment of partial tear ACL and remnant preservation ACL reconstruction.

Conclusion

The study results indicated that the lyophilized hUSC-EXs preserve the characteristics of exosomes and can improve chronically injured(8 weeks) ACL cells. Lyophilized hUSC-EXs could serve as effective and safe biomaterials that are ready to use at room temperature to enhance cell activity in patients with partial ACL tears and after remnant preservation ACL reconstruction.

Declarations

Ethics approval

The animal use protocol listed below has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC No.110232). The studied exosomes were purified from the culture medium of human umbilical cord stem cells (IRB approval No. A202205014),

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

HLL and SYL contributed to writing the manuscript, CJH and SHH contributed to the design of experiment and concept of study. YMK and SCW contributed to the experiment and data analysis, CCL was the main design of experiment and concept, and contributed to manuscript writing and revising. All authors read and approved the final manuscript.

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Figures

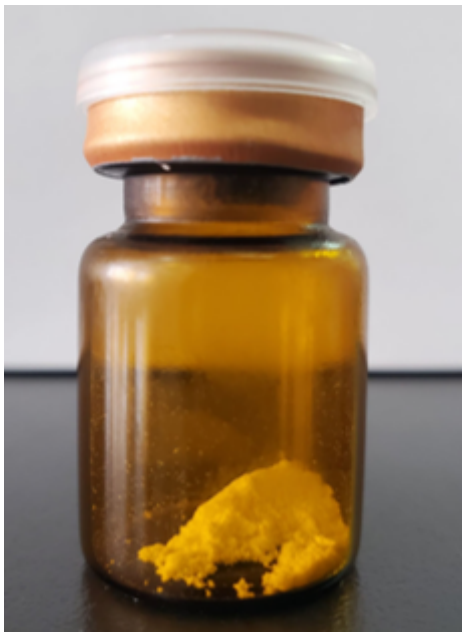

Figure 1

The hUSC-EX was lyophilized and aseptically stored in the bottle.

hUSC-EX: human umbilical cord stem cells derived exosome.

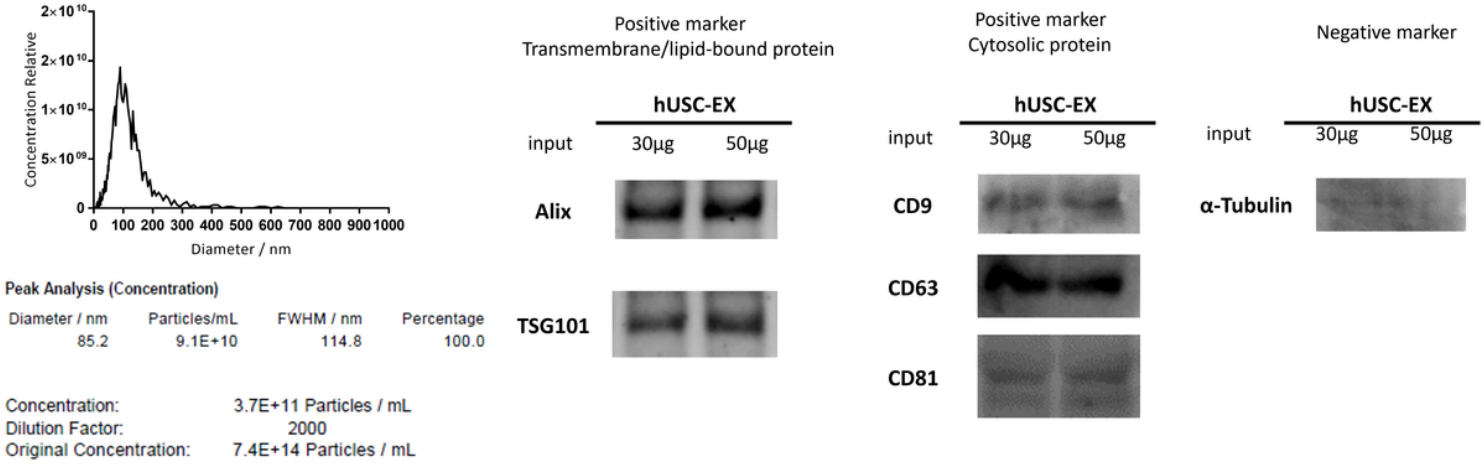

Figure 2

The size distribution and exosome markers of hUSC-EX.

hUSC-EX: human umbilical cord stem cells derived exosome.

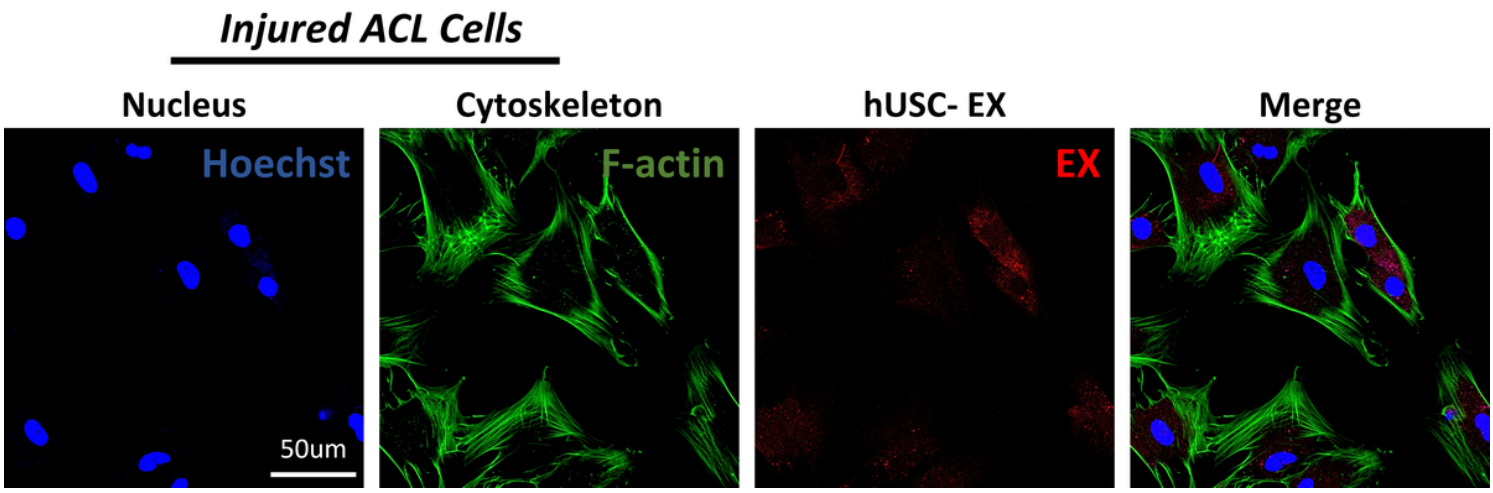

Figure 3

The immunofluorescence images showed hUSC-EX was uptaken and internalized by the injured ACL cells. ACL: anterior cruciate ligament; hUSC-EX: human umbilical cord stem cells derived exosome.

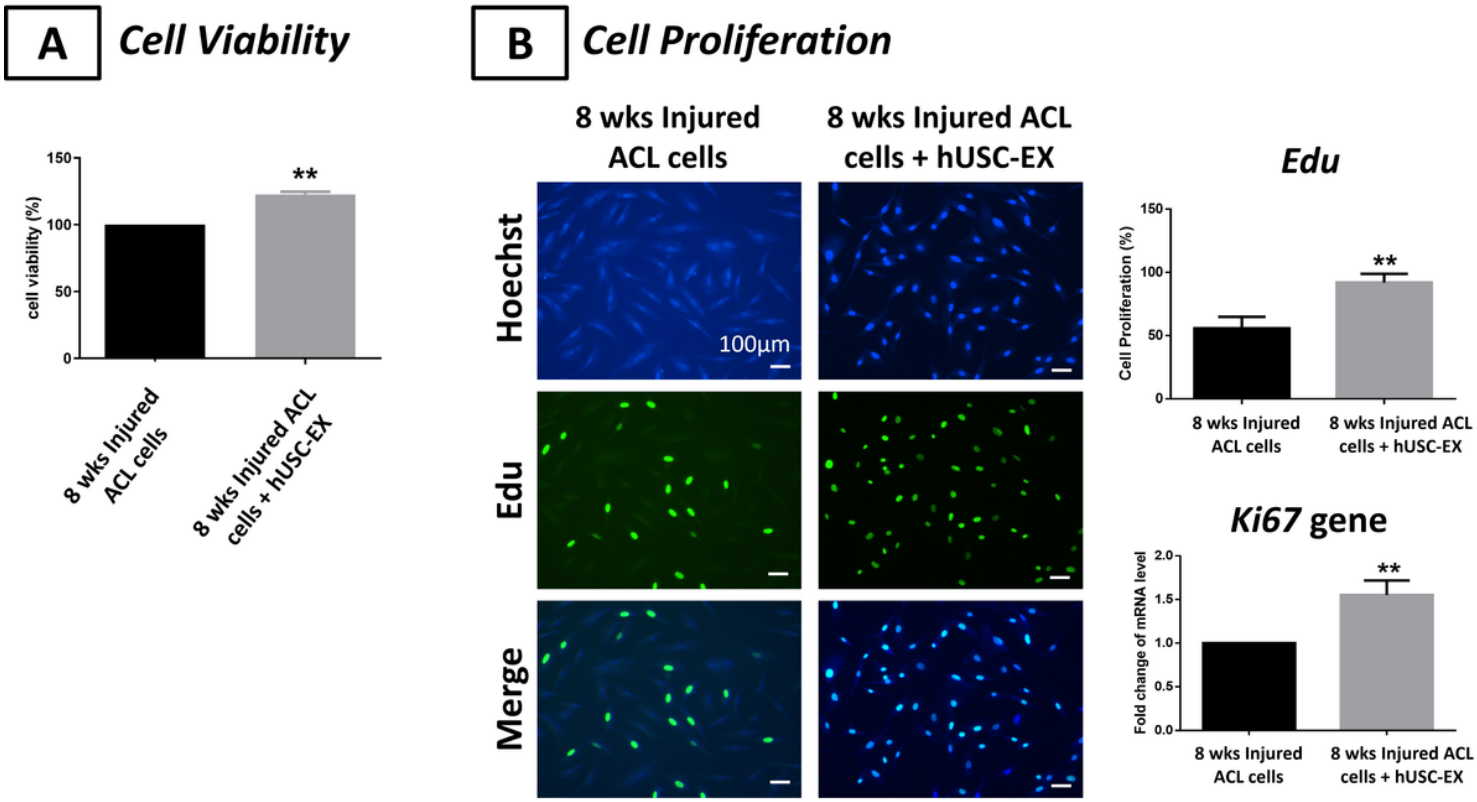

Figure 4

The cell viability and proliferation difference between the non-exosome treated 8 weeks injured ACL cells (control) and hUSC-EX treated cells. A) Cell viability (MTT); B) Cell proliferation (EdU, *Ki67* gene expression). hUSC-EX: human umbilical cord stem cells derived exosome.

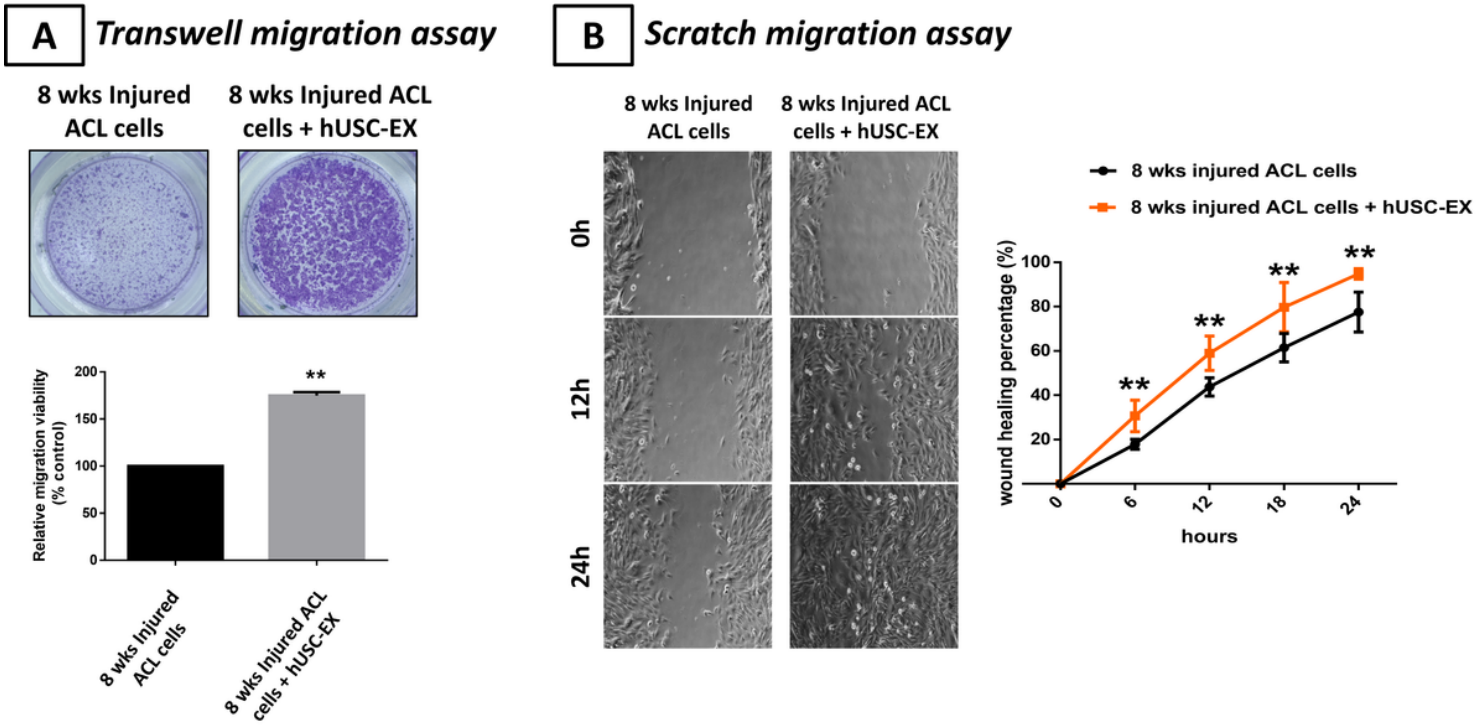

Figure 5

The migration capability difference between the non-exosome treated 8 weeks injured ACL cells (control) and hUSC-EX treated cells. A) Transwell migration assay; B) Scratch migration assay. hUSC-EX: human umbilical cord stem cells derived exosome.

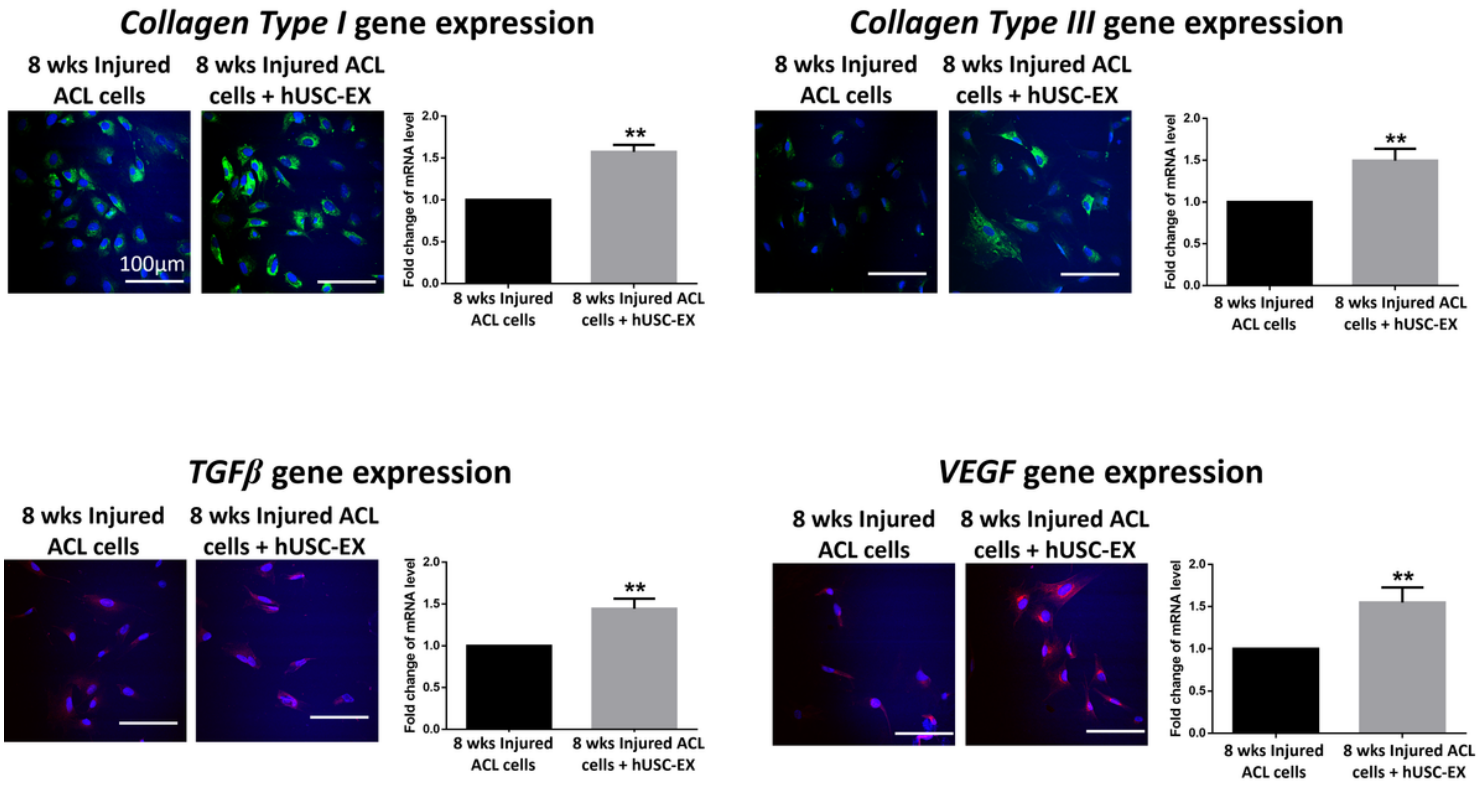

Figure 6

The expression of Collagen synthesis, *TGFβ*, and *VEGF* gene difference between the non-exosome treated 8 weeks injured ACL cells (control) and hUSC-EX treated cells. hUSC-EX: human umbilical cord stem cells derived exosome.

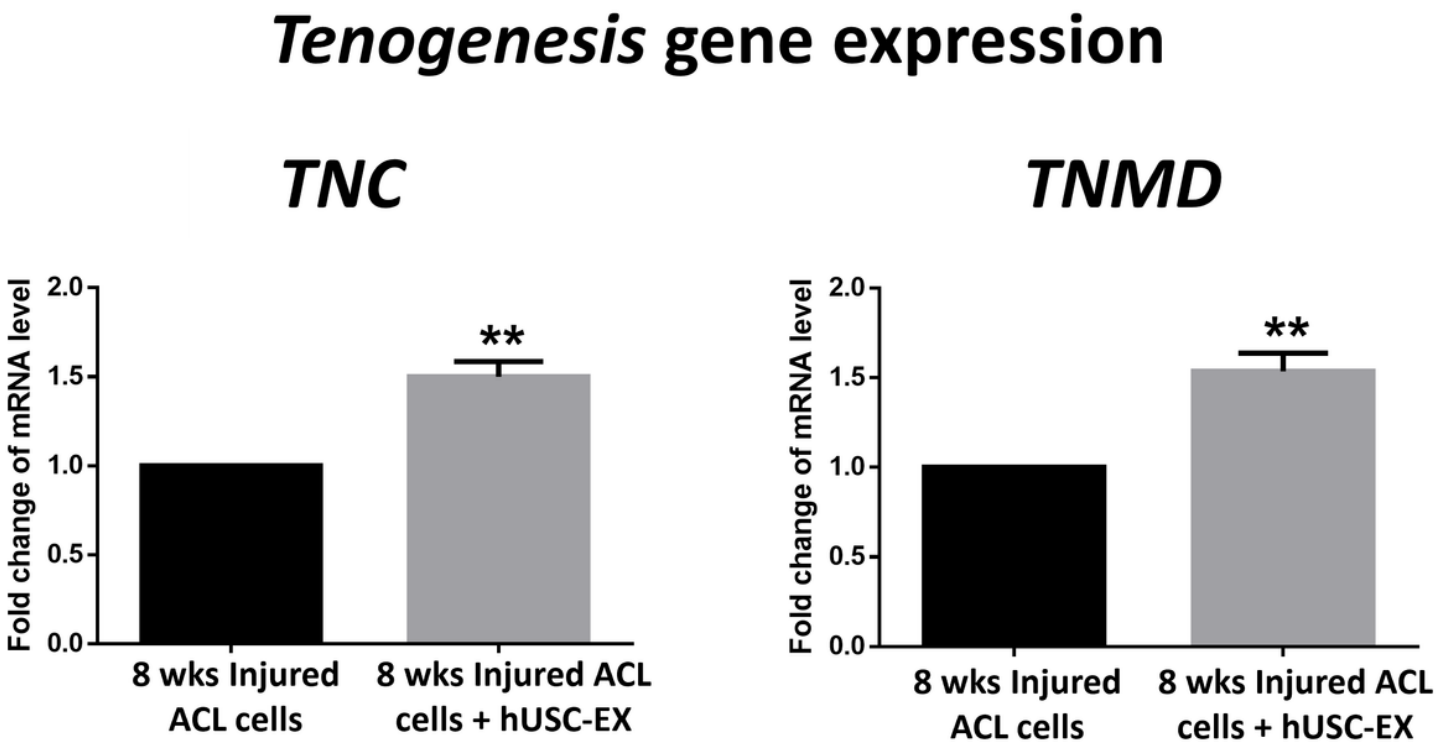

Figure 7

The expression of tenogenic markers (*TNC* & *TNMD*) gene difference between the non-exosome treated 8 weeks injured ACL cells (control) and hUSC-EX treated cells. hUSC-EX: human umbilical cord stem cells derived exosome.