Original Paper

Extracellular Vesicles Secreted by Human Urine-Derived Stem Cells Promote Ischemia Repair in a Mouse Model of Hind-Limb Ischemia

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Key Words

Extracellular vesicles • Urine-derived stem cell • Stem cell • Ischemia • Repair

Abstract

Background/Aims: Our previous studies have shown that human urine-derived stem cells (USCs) have great potential as a cell source for cytotrophy and tissue engineering and that extracellular vesicles (EVs) secreted by USCs (USCs-EVs) can prevent diabetes-induced kidney injury in an animal model. The present study was designed to evaluate the effects of USCs-EVs on ischemia repair. **Methods:** USCs-EVs were isolated and purified by a battery of centrifugation and filtration steps. The USCs-EVs were then characterized by transmission electron microscopy, western blot and tunable resistive pulse sensing techniques. After intramuscularly transplanting USCs-EVs into an ischemic mouse hind-limb, we observed the therapeutic effects of USCs-EVs on perfusion by laser doppler perfusion imaging, angiogenesis and muscle regeneration by histology and immunohistochemistry techniques over 21 days. We subsequently tested whether USCs-EVs can induce the proliferation of a human microvascular endothelial cell line HMEC-1 and a mouse myoblast cell line C2C12 by cell counting kit 8 assay *in vitro*. Meanwhile, the potential growth factors in the USCs-EVs and supernatants of the USCs cultures were detected by enzyme-linked immunosorbent assay. **Results:** The USCs-EVs were spherical vesicles with a diameter of 30–150 nm and expressed exosomal markers, such as CD9, CD63 and Tsg101. Ischemic limb perfusion and function were markedly increased in the hind-limb ischemia (HLI) model after USCs-EVs administration. Moreover, angiogenesis and muscle regeneration levels were significantly higher in the USCs-EVs treatment group than in the PBS group. The *in vitro* experiments showed that USCs-EVs facilitated HMEC-1 and C2C12 cell proliferation in a dose-dependent manner. **Conclusions:** These results revealed for the first time that USCs-EVs efficiently attenuate severe hind-limb ischemic injury and represent a novel therapy for HLI.

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Introduction

Mesenchymal stem cells (MSCs) have been proposed as an attractive candidate for cellular therapy in regenerative medicine, particularly in the treatment of ischemic diseases [1-4]. During the past several decades, MSCs have been successfully harvested from various tissues, such as bone marrow (BM), umbilical cord blood (UCB), umbilical cord (UC) tissue and adipose tissue (AT) [5-9]. However, loss of potency (proliferation and differentiation capability), limitations with respect to the availability of autologous cells (aging and age-related disorders), and the invasiveness of the procedures used for cell extraction have severely hampered the use of BM-, UCB-, UC- and AT-derived MSCs in clinical applications [10, 11].

We recently confirmed that a subpopulation of cells extracted from voided human urine known as urine-derived stem cells (USCs) have an MSC-like morphology, express MSC surface markers, and maintain a trilineage differentiation capacity *in vitro* [12, 13]. In addition, another team of researchers reported that USCs have the capability to differentiate into endothelial, urothelial and smooth muscle cells [14, 15]. In previous urological studies, USCs were used to construct tissue-engineered conduits for urinary diversion [15] and to ameliorate erectile dysfunction [16, 17]. Moreover, we found that USCs combined with bioactive materials accelerated skin-wound healing in rabbits [18] and that USCs can survive in and migrate to other parenchymal areas and differentiate into neuron-like cells in the rat brain [13].

The beneficial therapeutic effects of MSCs on different diseases appear to be derived from their ability to replace certain types of damaged or lost cells; recently, accumulating evidence supports the idea that MSCs exert therapeutic effects mainly via paracrine mechanisms [19, 20]. MSCs have been shown to release cytokines, growth factors, chemokines and extracellular vesicles, including endosome-derived exosomes. Exosomes are lipid bilayer membrane-bound vesicles released by almost all types of cells and contain a variety of functional proteins, lipids and RNAs [21-23]. In a previous study, we found that USCs-EVs prevent type I diabetes-induced kidney injury [24]. However, to our knowledge, the functional effects of either USCs-EVs or USCs on ischemic hind-limbs have not been evaluated.

Here, we tested the post-ischemic repair effects of USCs-EVs on mouse hind-limb ischemia (HLI) and elucidated the mechanisms underlying these effects. We found that USCs-EVs and USCs mitigate hind-limb ischemic injury by promoting angiogenesis and skeletal muscle regeneration both *in vivo* and *in vitro*. Our results suggest that USCs-EVs are useful as a treatment for ischemic diseases.

Materials and Methods

Cell culture

The indicated human microvascular endothelial cell line (HMEC-1) and mouse myoblast cell line (C2C12) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The HMEC-1 and C2C12 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, MO, USA) at 37°C in a humidified atmosphere containing 5% CO₂.

Isolation and purification of USCs-EVs

USCs were isolated from the urine of healthy adult donors, according to the methods described in our previous studies [13, 18]. The Ethics Committee of Shanghai Sixth People's Hospital Affiliated with Shanghai Jiaotong University approved the study, and written informed consent was obtained from all the volunteers upon their enrollment in the study. USCs-EVs were then isolated from USC culture supernatants by a series of centrifugation and filtration steps. Briefly, when the USCs reached approximately 80% confluency, they were washed twice with PBS and then cultured in serum-free MSC medium (StemRD, San Francisco, CA, USA) for an additional 48 h at 37°C with 5% CO₂. The conditioned medium was then collected and centrifuged at

300 × g for 10 min and 2000 × g for 10 min at 4°C and filtered through a 0.22-μm sterilized filter (Steritop™, Millipore) to remove any remaining cells and cellular debris. The supernatant was subsequently transferred to centrifugal filter devices (Amicon®, Millipore) and centrifuged at 3220 × g at 4°C until the volume in the upper compartment was reduced to approximately 200 μL. The ultrafiltrate was resuspended in PBS and then centrifuged at 100,000 × g for 70 min at 4°C (Sorvall, Avanti J-26XP, fixed angle rotor; Beckman Coulter, USA). This step was then repeated once, after which the USCs-EVs were stored in aliquots at -80°C until use.

Identification of USCs-EVs

Transmission electron microscopy (TEM) was performed to examine the morphology of the USCs-EVs. Briefly, fresh exosome samples were applied to a continuous carbon grid and negatively stained with 2% uranyl acetate for 60 seconds. The samples were then examined under an FEI Tecnai G2 Spirit Twin Transmission Electron Microscope (FEI™, Eindhoven, the Netherlands), and the images were captured using a digital camera (Olympus, Tokyo, Japan).

Western blotting analysis was used to identify the following USCs-EVs surface markers: CD9, Alix, and Tsg101. Protein from the exosome samples or cell lysates was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), and then incubated with the appropriate antibodies. Primary antibodies for CD9 (1:1000; Abcam, Cambridge, UK), CD63 (1:1000; Abcam), and TSG101 (1:1000; Santa Cruz, Dallas, USA) and a horseradish peroxidase (HRP)-conjugated secondary antibody were detected by enhanced chemiluminescence (Thermo Fisher Scientific, USA).

Tunable resistive pulse-sensing (TRPS) analysis was used to measure the concentrations and size distributions of the USCs-EVs. Nanopore (NP150; Izon Science, UK) was stretched to 47.00 mm with a voltage of 0.70 V. CPC100B particles (mean diameter: 114 nm, concentration: 1.0×10^{13} /mL; Izon Science, UK) were used to determine the sizes and concentrations of the exosomes, according to the manufacturer's instructions. All exosome samples were diluted with 0.22-μm filtered PBS, and >500 particles were counted in 5 min by a qNano platform (Izon Science, UK). Izon Control Suite software v2.2 was used for data processing and analysis.

Mouse HLI model and groupings

All animal study protocols were approved by the Animal Research Committee of Shanghai Sixth People's Hospital (SYXK (Shanghai, China) 2011-0128, 1 January 2011). HLI was induced in male C57BL/6 mice aged 10-12 weeks after the mice were anesthetized with 4% chloral hydrate (9 mL/kg) via intraperitoneal injection. The left femoral artery, which originates beneath the inguinal ligament and eventually splits into the saphenous and popliteal arteries, was subsequently excised. The mice were then randomly assigned to 4 different groups (n=15 for each group), which were intramuscularly injected with 100 μL of PBS (the PBS group), 1×10^6 USCs in 100 μL of PBS (the USC group), or USCs-EVs in 100 μL of PBS (the USCs-EVs group) via four sites of the gracilis muscle in the medial thigh as previously described [25, 26]. EVs secreted by induced pluripotent stem cell-derived MSCs (iMSCs-EVs) ameliorated limb ischemia in our previous study [27]; hence, mice injected with iMSCs-EVs in 100 μL of PBS (the iMSCs-EVs group) were used as a positive control. The concentration used in both the USCs-EVs and iMSCs-EVs groups was 2×10^{10} particles/mL. The mice in the USC group were immunosuppressed with oral cyclosporine (Neoral; Novartis, China). In addition, 10 mice (the Sham group) underwent a sham operation in which the femoral artery was dissociated but was not excised.

Blood flow restoration assessment and limb function score

Non-invasive measurements of hind-limb blood flow were performed by a Laser Doppler perfusion imager (Moor Instruments, Devon, UK) at 7, 14, and 21 days after femoral artery excision. Digital color-coded images were used to quantify hind-limb tissue perfusion. The difference in blood flow between the ischemic and non-ischemic limbs was expressed as the ratio of blood flow in the ischemic limb to blood flow in the non-ischemic limb. Moreover, an ambulation score (0 = flex toes to grasp cage; 1 = plantar flexion; 2 = no foot dragging but no plantar flexion; 3 = foot dragging) and necrosis score (1 to 5 = one to five fingernails damaged, 6 to 10 = one to five fingers fully damaged, 11 = paw fully damaged) were used to grade the degrees of hind-limb impairment and ischemic damage, as described previously [28, 29].

Histology and immunohistochemistry

For histological analysis, the hind-limb muscles of the mice were removed at a designated time, fixed in 10% formalin, routinely dehydrated with an alcohol gradient, embedded in paraffin and serially sectioned at a thickness of 5 μ m. Hematoxylin and eosin (H&E) staining was used for morphological and muscle regeneration (rounded fibers with centrally located nuclei) analyses. For immunofluorescence assay, the gracilis muscles were fixed in 4% paraformaldehyde, embedded in optimal cutting temperature (OCT) compound, snap-frozen in liquid nitrogen, and maintained at -80°C . The frozen tissue sections (8- μ m thickness) were blocked in 1% BSA for 30 min at room temperature (RT), incubated with rabbit anti-CD31 (1:100, Abcam) and rabbit anti-CD34 (1:150, Abcam) antibodies overnight at 4°C , and then incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:200, Jackson) for 1 h at RT before being counterstained with 4',6-diamidino-2-phenylindole (DAPI, Beyotime). All images were captured with a Leica microscope. Moreover, the images were analyzed by two pathologists who were unaware of the group assignments using Image Pro Plus software.

In vitro cell proliferation assay

Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. HMEC-1 and C2C12 cells were seeded in 96-well plates at a density of 3000 cells per well. The cells were then cultured with 100 μ L of DMEM containing different concentrations of USCs-EVs (0, 1×10^9 and 5×10^8 particles/mL). CCK-8 solution (10 μ L/well) was added to the medium, after which the cultures were incubated for an additional 2 h at 37°C . The absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). All experiments were performed in triplicate and were repeated three times.

Enzyme-linked immunosorbent assay (ELISA)

According to the manufacturer's instructions, the concentrations of vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), and hepatocyte growth factor (HGF) were verified by commercial ELISA kits (Westang, China) in sonication-disrupted USCs-EVs and supernatants of the USCs cultures. Fresh culture medium was used as a control, and three independent experiments were performed.

Statistical analysis

All data are presented as the mean $\pm$ S.E.M. SPSS 19.0 (SPSS Institute, Chicago, IL, USA) was used to perform the data analysis with one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test. $P < 0.05$ was considered statistically significant.

Results

Characterization of USCs-EVs

USCs-EVs were collected from USCs according to the steps described in the Materials and Methods. TEM showed that the USCs-EVs were spherical vesicles with a diameter of 30–100 nm (Fig. 1A). Western blotting analysis indicated that the exosome-specific markers CD9, Alix, and TSG101 were expressed in the USCs-EVs (Fig. 1B). TPRS analyses demonstrated that the majority of the USCs-EVs were ≤ 150 nm in size and that the concentration of the USCs-EVs was approximately 2×10^{10} particles/mL (Fig. 1C).

Improvement in perfusion and limb function after USCs-EVs transplantation

To assess the impact of local USCs-EVs injection on blood flow restoration in the limb ischemia model, we used laser Doppler flow imaging (LDPI) to measure tissue perfusion on days 0, 7, 14, and 21 after surgery. The left ischemic limbs were poorly perfused compared with the right normal limbs in each group of mice on day 0 after surgery; however, circulation was progressively restored over a period of 21 days (Fig. 2A). Each group of ischemic mice displayed reduced perfusion until 7 days after surgery; however, perfusion was significantly improved both in the USCs-EVs group and the positive control (iMSCs-EVs) group compared with the PBS group on postoperative day 14 (Fig. 2B; $P < 0.05$). Perfusion in the USCs-EVs

Fig. 1. Characteristics of the USCs-EVs. (A) USCs-EVs morphology, as demonstrated by TEM, scale bar = 100 μ m. (B) Western blot analysis of exosomal CD9, ALIX, and TSG101 protein expression in USCs-EVs. (C) TRPS analysis of the concentrations and size distributions of the USCs-EVs and iMSCs-EVs.

Fig. 2. Accelerated recovery of perfusion and limb function after USCs-EVs transplantation. (A) LDPI showing dynamic changes in perfusion in the ischemic limb on days 0, 7, 14, and 21 after surgery. (B, C and D) note that the legend is the same for all graphs: (B) The ratio of perfusion in the ischemic (left) limb to perfusion in the non-ischemic (right) limb at the indicated time points. Ambulatory impairment scores (C) and foot necrosis scores (D) were evaluated in each group of mice. Data are presented as the mean $\pm$ SEM, statistical significance: * $P < 0.05$.

and iMSCs-EVs groups increased remarkably to a level that was approximately 80 percent of normal on day 21 after surgery (Fig. 2B; $P < 0.05$). However, we noted no significant difference in perfusion between the USC and USCs-EVs groups on day 14 or 21 after surgery. The PBS group displayed a lower perfusion ratio than the other groups, and perfusion in the ischemic limb did not reach one-half of that in the contralateral normal limb in the PBS group until 21 days after surgery. However, the perfusion ratio remained stable and within the normal range in the sham group (Fig. 2B). These data suggest that the injection of both USCs-EVs and USCs plays an important role in restoring perfusion.

To evaluate the functional recovery of the ischemic limb, we scored limb ambulatory impairment and tissue damage. Active use of the hind-limb was clearly reduced in the ischemic limb compared with the normal limb during the first 3 days after surgery but gradually improved after 7 days post-surgery. The ambulatory impairment and foot necrosis scores were strikingly lower in the USCs-EVs, USCs and iMSCs-EVs groups than in the PBS group on day 21 post-surgery (Fig. 2C and 2D; $P < 0.05$), findings similar to those noted in the perfusion analyses. These results indicated that USCs-EVs and USC transplantation exerted beneficial effects with respect to the recovery of ischemic limb function. In addition, we noted no remarkable difference in the recovery of ischemic limb function between the USC and USCs-EVs groups on days 7, 14, or 21 post-surgery (Fig. 2C and 2D).

Advancement of angiogenesis after USCs-EVs injection

To assess neovascularization, we measured the capillary density (the number of microvessels per square millimeter) of the ischemic limb on postoperative days 14 and 21. We performed immunofluorescence staining for the endothelial markers CD31 and CD34 to observe angiogenesis in each group of mice. As shown in Fig. 3A, representative images of CD31 and CD34 staining revealed that capillary density was increased in all the treatment groups compared with the PBS group on days 14 and 21 post-surgery. Capillary density gradually increased with the passage of time. The CD31 staining results showed that capillary density was 2.7-, 2.5- and 2.6-fold higher in the USC, USCs-EVs and iMSCs-EVs groups than

Fig. 3. USCs-EVs injection facilitated angiogenesis. (A) Representative images of ischemic gracilis muscles stained for CD31 and CD34 in each group of mice. Capillary density was determined by immunofluorescence staining for CD31 or CD34 (red) and nuclear staining with DAPI (blue). Scale bars = 100 μ m. (B and C) note that the legend is the same for all graphs: quantitative analysis of capillary density by CD31 (B) and CD34 (C) in each group of mice. Data are presented as the mean $\pm$ SEM, statistical significance: * $P < 0.05$.

Fig. 4. Improvement in muscle regeneration after treatment with USCs-EVs. (A) Representative photomicrographs of H&E-stained gracilis tissue cross-sections from ischemic limbs at 14 and 21 days after surgery. Scale bars = 50 μ m. (B and C) note that the legend is the same for all graphs: the number (B) and diameter (C) of centrally located nuclei in regenerating muscle fibers were quantified in each group of mice. Data are presented as the means $\pm$ SEM, statistical significance: * $P < 0.05$.

in the PBS group, respectively, on day 14 (Fig. 3B; $P < 0.05$). In addition, capillary density was 3.1-, 3.0- and 3.1-fold higher in the USC, USC-EVs and iMSC-EVs groups than in the PBS group, respectively, on day 21 (Fig. 3B; $P < 0.05$). The CD34 staining results showed that the mice in the treatment groups had a significantly higher capillary density than those in the PBS group on days 14 and 21 (Fig. 3C; $P < 0.05$). These results suggest that USCs and USC-EVs, similar to iMSC-EVs, facilitate vascular regeneration in ischemic limbs. However, there was no significant difference in capillary density between the USC and USC-EVs groups.

Enhancement of muscle regeneration after USCs-EVs transplantation

To determine the effect of USCs-EVs on injured muscle fibers in ischemic limbs, we performed H&E staining to analyze muscle regeneration (muscle fibers with centrally located nuclei) and myofiber diameter in tissue cross-sections. As shown in Fig. 4A, the normal myofibers had a regular structure, and the nucleus was located under the sarcolemma, whereas the myofibers in the PBS group showed obvious metaplasia, as well as morphological malformations and myofiber loss. The number of myofibers with centrally located nuclei was approximately 50% higher in the treatment groups than in the PBS group on days 14 and 21 after injury (Fig. 4B; $P < 0.05$). The numbers of regenerated muscle fibers had increased by 27.6%, 32.9% and 34.8% in the USC, USC-EVs and iMSC-EVs groups compared with the PBS group, respectively, on day 14 after surgery (Fig. 4B; $P < 0.05$). In addition, the mean diameters of the muscle fibers were quantitatively larger in the treatment groups than in the PBS group on days 14 and 21 (Fig. 4C; $P < 0.05$). These data indicate that USC and USC-EVs transplantation facilitates the repair of ischemic hind-limb muscles; what's more, there

Fig. 5. Pro-proliferative effect of USCs-EVs on endothelial cells and myoblasts. (A and B) note that the legend is the same for all graphs: HMEC-1 (A) and C2C12 cell proliferation (B) was measured by CCK8 assay on days 1, 2, 3 and 4 post-incubation with different concentrations of exosomes. 10^9 indicated that the exosome concentration was 1×10^9 particles/mL, and 10^8 indicated that the exosome concentration was 5×10^8 particles/mL. Data are presented as the mean $\pm$ SEM, statistical significance: * $P < 0.05$.

Fig. 6. ELISA-based assay for detection of VEGF, HGF and IGF-1 in USCs-EVs. (A, B and C) note that the legend is the same for all graphs: the concentrations of VEGF (A), HGF (B), and IGF-1 (C) were measured by ELISA in fresh culture medium (Control), supernatants of the USCs cultures (Supernatants) and sonication-disrupted USCs-EVs (USCs-EVs). Data are presented as the mean $\pm$ SEM, statistical significance: * $P < 0.05$.

was no significant difference in mean muscle fiber diameter between the USC and USCs-EVs groups.

USCs-EVs promote cell proliferation *in vitro*

To test the *in vitro* effect of USCs-EVs on cell proliferation, we performed CCK-8 assay. As shown in Fig. 5A and 5B, the proliferative capacity of the HMEC-1 and C2C12 cells in the EVs treatment groups was significantly higher than that of the HMEC-1 and C2C12 cells in the control group at the above time points ($P < 0.05$). The effects of the above treatments were dose-dependent. In addition, no significant difference in the absorbance was observed between the USCs-EVs and iMSCs-EVs groups when the same treatment concentrations were used.

USCs-EVs contain angiogenesis- and myoblast proliferation-related growth factors

To identify the possible mechanisms by which the USCs-EVs increased angiogenesis and C2C12 cell proliferation, we detected the levels of the growth factors VEGF, HGF and IGF-1 by ELISA. As shown in Fig. 6, VEGF, HGF and IGF-1 levels were markedly higher in the USCs-EVs and supernatants of the USCs cultures than in the control samples ($P < 0.05$).

Discussion

This study represents the first exploration of the therapeutic effect of USCs-EVs and USCs on HLI in a murine model. Interestingly, we found that both USCs-EVs and USCs protect mouse hind-limbs against ischemic injury. Our results show that USCs-EVs and USCs significantly increased angiogenesis and skeletal muscle regeneration both *in vivo* and *in vitro*. These findings have, to some extent, deepened our current understanding of USCs-EVs and USCs and suggest that they represent a new therapy for ischemic disease.

MSCs have drawn much attention in experimental and clinical studies in the field of cellular therapy for ischemic diseases. MSCs dramatically reduced infarct volumes, improved behavioral outcomes and protected neuronal function in animals with acute ischemic stroke [30-32]. MSC transplantation significantly decreased necrotic myocardial areas and improved left ventricular remodeling following myocardial infarction (MI) [3, 33]. In addition, systematically administered MSCs reportedly accelerated kidney functional

recovery after ischemia-reperfusion (IR) injury [34, 35]. Similarly, in this study, the injection of USCs significantly attenuated HLI in a murine model. USCs can be obtained using a very safe, simple, low-cost and noninvasive procedure that does not require enzyme digestion. Prior studies have shown that USCs, similar to iMSCs, have a fast, stable expansion speed and maintain telomere length and high telomerase activity [36, 37]. Therefore, USCs may represent a virtually unlimited supply of autologous cells that may be used for cell therapy in rodent models or clinical trials. However, Jeong et al [38], found that malignant tumors formed following the injection of BM-MSCs cultured over a short period of time into the peri-infarct tissues of mice with acute MI or the hind-limb muscles of mice with diabetic neuropathy. Although the tumorigenicity of USCs is currently unknown, we found it useful to monitor for epigenetic changes and alterations in mitochondrial status during the *in vitro* expansion of the cells.

A new cell-free therapy that uses stem cell-derived exosomes for tissue repair has recently been widely studied [39, 40]. In this study, we observed the protective effect of USCs-EVs (2×10^9 particles) on hind-limb ischemic injury. In addition, MSCs have been successfully generated from iPSCs generated from adult somatic cells (e.g., fibroblasts) using previously described reprogramming techniques [41]. We found that iMSCs-EVs alleviated hind-limb ischemic injury [27], hepatic IR injury [42] and osteoarthritis [43]. Moreover, iMSCs-EVs also prevented osteonecrosis of the femoral head [44] and enhanced bone regeneration [45, 46] and cutaneous wound healing [47]. Based on these findings, we used iMSCs-EVs particles administered at the abovementioned concentration as a positive control in experiments designed to detect the effects of the USCs-EVs on ischemic hind-limb repair. In the present study, the post-ischemic intramuscular administration of USCs-EVs had active effects on the functional recovery of ischemic hind-limbs similar to those of the administration of iMSCs-EVs, as the treatment promoted angiogenesis and muscle regeneration.

There is no doubt that angiogenesis plays a critical role in the repair of ischemic tissue. Endothelial cells (ECs) are the essential elements of new vessels, and the enhancement of EC proliferation is the premise for angiogenesis. In this study, we found that USCs-EVs promoted HMEC-1 proliferation in a dose-dependent manner. The same concentrations of USCs-EVs and iMSCs-EVs exhibited similar therapeutic potential *in vitro*. Quiescent muscle satellite cells (mSCs) become activated in response to muscle injury and actively proliferate to form myoblasts, which are the primary cell type involved in muscle regeneration [48, 49]. The vital mechanism underlying proliferation and differentiation in mSCs is highly conserved in both primary and C2C12 myoblast cultures *in vitro* [50]. Myoblast differentiation is indispensable for muscle regeneration; however, the initial burst of myoblast proliferation is also essential for this process [51, 52]. In this study, we observed that both USCs-EVs and iMSCs-EVs promoted C2C12 myoblast proliferation. Previous studies reported that VEGF, HGF and IGF-1 were not only potential proangiogenic factors but also promoted myoblast proliferation [53-59]. In the present study, we found that VEGF, HGF and IGF-1 were loaded in the USCs-EVs.

Exosome-mediated therapy offers several advantages over conventional stem cell therapy. First, exosomes play an important role in cell-cell communication by transferring contained types of bioactive molecules, including proteins, lipids and nucleic acids [60]. Second, nanosized exosomes can easily penetrate through various biobarriers, including the blood-brain barrier [61]; thus, exosomes enable the treatment of previously inaccessible disease foci. For instance, systemically administered MSC-derived exosomes promoted stroke functional recovery by enhancing angiogenesis and neurogenesis [62]. Last but not least, large numbers of young and vigorous USCs at low passages can be acquired easily; thus, we can produce many USCs-EVs within a short period of time. Moreover, exosomes can be used as a drug delivery vessel. Pascucci et al [63], demonstrated that MSC-derived exosomes have the capacity to package and deliver paclitaxel to target cells. In addition, engineered exosomes coupled to rabies virus glycoproteins [64] or iRGD (CRGDKGPDC)-Lamp2b [65] showed a higher efficiency with respect to targeting special cells than other exosomes. However, it is not known whether there is an optimal therapeutic concentration

for exosomes. Future studies will undoubtedly address this important issue. Moreover, identifying the molecular constituents of USCs-EVs, including miRNAs, and proteomics analyses will be beneficial for studying the mechanisms by which exosomes promote angiogenesis and muscle regeneration.

In conclusion, this study showed that the post-ischemic intramuscular administration of USCs-EVs exerted therapeutic effects at least in part by facilitating angiogenesis and muscle regeneration in a mouse model of HLI. USCs-EVs, as well as iMSCs-EVs, which are available in an “off-the-shelf” format, may be the executors of the biological activity of USCs and iMSCs in the treatment of tissue ischemia.

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Disclosure Statement

The authors declare that they have no financial or commercial conflict of interests.

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