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SURGICAL TECHNIQUES

A Technique for Systemic Mesenchymal Stem Cell Transplantation in Newborn Rat Pups

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ABSTRACT

Mesenchymal stem cells (MSC) have the potential to aid tissue regeneration. Intravenous (IV) MSC administration is currently being assessed following tissue injury. However, few studies have been performed to establish a safe and effective method of IV MSC infusion for newborns. We have established a safe, nontraumatic and effective technique for systemic MSC transplantation in newborn rats. Yellow-fluorescent-protein (YFP)-labeled MSC were characterized using MSC markers and their differentiation potential was confirmed. Rat pups were delivered by C-section on gestational day 21. The umbilical vein (UV) was cannulated and used for IV injection of MSC or saline control, which was performed under ultrasonographic imaging. An additional control group consisted of UV MSC injection in adult mice. Mean operating time, success rate of cannulation and death rate were recorded. YFP-MSC quantification in multiple organs was performed. Mean operating time was 3.9 ± 1.1 min. The success of UV MSC injection was 92.8%. The immediate and 24 hr delayed death rate for rat pups was significantly lower than that of adult mice ($p < .05$). No pups receiving saline injection died. After locating the patent foramen ovale (PFO) of newborn pups by ultrasonographic imaging, extra pulse-waves and wave-shape changes were detected when MSC were injected. The number of YFP-MSC was 15.8 ± 4.1 cells per visual field (CPVF) in the lungs, 2.9 ± 1.2 CPVF in the heart, and 19.8 ± 5.0 CPVF in the intestines. We conclude that IV MSC infusion through the UV is a convenient, safe, and effective method for systemic MSC transplantation in prematurely delivered newborn rats.

Keywords: mesenchymal stem cells; cell transplantation; newborn; intravenous; umbilical vein; rat model

INTRODUCTION

Mesenchymal stem cells (MSC) have the ability to differentiate into different cell lineages [1–3] and can stimulate wound healing via paracrine signaling pathways [4, 5]. Preclinical studies have shown that MSC can regulate the host immune response, thus avoiding recognition and subsequent rejection by recipients [6]. Local stem cell (SC) delivery may result in increased risks of bleeding and tissue injury when administered by intralesional injection, and occlusion when administered intra-arterially [7–9]. Intravenous (IV) infusion has been used for systemic SC delivery in preclinical studies [5, 10] and in clinical trials [11–14]. However, it has been noted that a large fraction of systemically infused MSC become trapped in the lung [15]. Thus, pulmonary passage is a major obstacle for IV SC delivery [16]. This pulmonary first-pass effect not only causes poor efficiency of MSC delivery, but it also threatens the life of experimental animals [17]. Pulmonary sequestration by MSC intravascular infusion causes mortality from 25% to 40% [18].

Here, we introduce a technique for systemic MSC transplantation in newborn rat pups immediately after birth. This technique is easy to perform, has a short operating time, a high success rate, high efficiency of systemic MSC delivery, and a low mortality rate.

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MATERIALS AND METHODS

Ethics Statement

All animals received humane care in accordance with the requirements of Institutional Animal Care and Use of the Committee (IACUC) at the Research Institute at Nationwide Children's Hospital. Veterinarians skilled in the healthcare and maintenance of rodents supervised animal care. Reasonable efforts were made to minimize suffering of animals. The use of animals was minimized by using an experimental design that permitted statistically-significant changes to be demonstrated with the smallest number of animals per group and the smallest number of groups, which was consistent with scientific rigor.

Culture of Murine Bone Marrow-Derived MSC and Preparation for Injection

Murine yellow fluorescence protein (YFP)-labeled bone marrow-derived MSC (YFP-BM-MSC) at passage 2 were kindly provided by Research Institute at Nationwide Children's Hospital, Columbus, Ohio [19]. These cells were initially derived as follows. A transgenic construct (pCX::EYFP) containing an enhanced YFP gene under the control of a chicken beta actin promoter coupled with the cytomegalovirus (CMV) immediate early enhancer, was introduced into (129×1/SvJ × 129S1/Sv) F1-derived R1 mouse embryonic stem (ES) cells. The homozygotes [129-Tg (CAG-eYFP) 7AC5Nagy/J, http://jaxmice.jax.org/strain/005483.html] were used as the source of BM-MSC. BM was harvested from the femurs and tibias of hind limbs and suspended in Dulbecco's Modified Eagle Medium (D-MEM) Nutrient Mixture F-12/GlutaMAX-I^{TM} medium (GIBCO Invitrogen; Carlsbad, CA). The cell mixture was pipetted and filtered through a cell strainer with 70 μm nylon mesh (Becton Dickinson; Franklin Lakes, New Jersey; http://www.bd.com), and seeded in D-MEM Nutrient Mixture F-12/GlutaMAX-I^{TM} medium supplemented with 10% MSC-qualified fetal bovine serum (FBS) (GIBCO, Grand Island, NY) and 0.01% gentamicin (GIBCO, Grand Island, NY) at 37°C in 5% CO_2. Culture medium was changed every four days and nonadherent cells removed. Prior to MSC injection, adherent cells were trypsinized (0.25% trypsin, Cellgro, Manassas, VA) for 3 min and then D-MEM/F12/GlutaMAX-I^{TM} medium supplemented with 10% MSC-qualified FBS was added to neutralize the trypsin. Cells were quantified using a hemacytometer and centrifuged at 800 rpm for 5 min at 4°C. Supernatants were discarded and pellets were resuspended in sterile saline. Suspended MSC were filtered through a cell strainer with 70 μm nylon mesh before injection. The concentration of MSC was adjusted to 7.5×10^6 cells/ml for injection. MSC suspensions were loaded into 0.3 ml low-dose U-100 insulin syringes with 29 gauge needles (Becton Dickinson; Franklin Lakes, New Jersey). Prior to IV infusion, syringes were maintained at 4°C with continuous shaking and MSC gently resuspended to ensure they were not aggregated prior to infusion.

Vimentin Immunocytochemical Staining of MSC

Vimentin is the main intermediate filament protein in mesenchymal cells and is therefore considered as a positive marker for MSC. Vimentin immunocytochemistry was performed as follows: Cultured MSC were fixed in 4% paraformaldehyde (USB Corporation; Cleveland, OH) at 4°C for 20 min and rinsed in phosphate-buffered saline (PBS) (Cellgro, Manassas, VA) three times. Cells were then incubated with mouse antiVimentin monoclonal antibody at a 1:50 dilution (Thermo Scientific; IL, USA; http://thermoscientific.com/ab) for 2 hr at room temperature, rinsed with PBS three times, and incubated with Cy3 labeled donkey antimouse antibody at a 1:500 dilution (Jackson ImmunoResearch, West Grove, PA) for 1 hr at room temperature. Counterstaining of nuclei was accomplished using 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI). Fluorescence was observed under a fluorescent microscope (Axioscope, Carl Zeiss; Jena, Germany) using green fluorescence protein (GFP), Cy3, and DAPI channels.

MSC Differentiation Assay

To confirm the ability of MSC to differentiate, cells were grown in adipogenic or osteogenic differentiation media for 15 days (Adipogenic and Osteogenic Differentiation kits, GIBCO Invitrogen, Grand Island, NY). MSC cultured without adipogenic or osteocyte differentiation media were used as undifferentiated controls. For osteogenic differentiation, a 60%∼80% subconfluent culture of MSC from passages 2 to 4 was used, and osteogenic differentiation medium was replaced twice a week. After 15 days in differentiation medium, Alizarin Red S solution (pH 4.2) was added to the cultures for 3 min and then removed. For adipogenic differentiation, MSC were grown in adipogenic differentiation medium that was replaced twice a week, with Oil Red O staining performed 15 days later.

Cesarean section (C-section) Rat Pup Delivery

Time pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) underwent C-section under CO_2 anesthesia on day 21st of gestation. After delivery, placentas were kept moist and warm, and the integrity of the umbilical cords maintained for injection. The

premature newborn rats (average weight 5.2 g) were placed in a neonatal incubator for temperature control. Ninety-four rat pups were delivered and used for experimentation.

Intravenous (IV) Cannulation and Infusion of MSC in Newborn Rat Pups

Immediately following C-section, newborn rat pups were anesthetized with inhalational isofluorane in 4% O_2. The placenta was placed on a gauze pad and the umbilical cord straightened for exposure. Under a surgical dissecting microscope, the membrane covering the umbilical vein (UV) and arteries was dissected and the vein separated from the arteries. A fine-toothed forceps was placed beneath the exposed UV. An oblique incision ($\sim$1.5 mm) was made in the UV and the vein was flushed with saline. One end of the tip of a piece of polyethylene-10 (PE-10) tubing (Becton Dickinson, Sparks, MD) was slightly stretched to make it thinner, and the other end of the tubing was fitted onto the needle of the syringe holding the MSC suspension. Using sterile technique, the stretched end of the tube was cannulated into the UV and the tube was fixed with an atraumatic vessel clip (Figure 1). A total volume of 40 μl containing 300×10^3 MSC was infused through the UV of rat pups ($N = 83$). MSC suspensions were injected within 1 min of cannulation. Rat pups receiving the same volume of IV saline injection were used as control animals ($N = 11$). Injections that drove blood in the UV back to the circulation were considered to be successful. Fluid extravasation, UV rupture, resistance while injecting or obstruction of UVs were considered to be signs of injection failure. Mean operating time for each successful cannulation and injection was recorded.

Intravenous (IV) Infusion of MSC in Adult Mice

In an effort to compare IV MSC infusion in adult animals compared to newborn animals, we chose to use adult mice since their bodyweight was approximately five times that of newborn rats, as opposed to using adult rats that would have a bodyweight of $\sim$50 times that of newborn rats. FVB mice (12 weeks of age) were anesthetized with inhalational isofluorane in 4% O_2 and 100 μl of a suspension of 1×10^6 MSC was infused with a 28 gauge needle through the tail vein using a dissecting microscope. This concentration of MSC was calculated to be comparable to the concentration of MSC administered to rat pups based on body weight. Mice receiving saline injection served as negative controls.

Intravenous (IV) Injection of Methylene Blue into Cannulated UVs

A separate group of pups were injected with methylene blue dye to confirm that the injected fluid entered the systemic circulation after injection. A volume of 40 μl of methylene blue was injected via the tubing cannulating the UV. In these animals, the abdominal wall was open in order to track the methylene blue in the organs inside the abdominal cavity.

Ultrasonographic Scanning of Right-to-Left Shunt in Newborn Rat Pups

In vivo cardiac structures were identified using a VisualSonics Vevo 2100 with a 40 MHz transducer (Visualsonics, Toronto, Ontario). After UV cannulation, rat pups were moved to a heated procedure board. Next, pre-warmed ultrasound gel (Aquasonic, Parker Labs, Farifield, New Jersey) was placed on the chest and a 15 MHz probe (optimized and dedicated to rodent studies) was placed in a subcostal orientation and a four chamber apical view was obtained. After obtaining the four chamber view, the patent foramen ovale (PFO) was visualized. Subsequently, the sample volume was injected to the level of the PFO and pulsed wave Doppler was used to capture baseline shunt flow. When injecting MSC suspensions, extra waves and changes of wave shapes were recorded.

Monitoring of Animals after IV MSC Infusion

Adult mice ($N = 23$) were monitored for 24 hr after IV MSC infusion. Prematurely delivered newborn rats ($N = 77$) were transferred to surrogate rat mothers and monitored for 96 hr. Immediate deaths and deaths within 24 hr after MSC injection were recorded.

Quantification of MSC Engraftment

After 96 hr, 11 of the rat pups that received systemic MSC administration and 11 control rat pups that received saline injections were euthanized by carbon dioxide asphyxiation followed by exsanguination. Lungs, hearts, and intestines were harvested and fixed in fixation solution containing 1% paraformaldehyde, 15% picric acid, and 0.1 M sodium phosphate buffer (pH 7.0) and shaken gently at 4°C overnight. Samples were embedded in Tissue-Tek Optimal Cutting Temperature (OCT) (Sakura Finetek, Torrance, CA) compound and frozen sections (10 μm) made. Slides were washed with PBS three times and mounted with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA). Fluorescence was observed under a fluorescence microscope (Axioscope, Carl Zeiss; Jena, Germany) using GFP and DAPI channels. Quantification of MSC was performed by counting YFP-positive cells per visual field (CPVF) at 100× magnification.

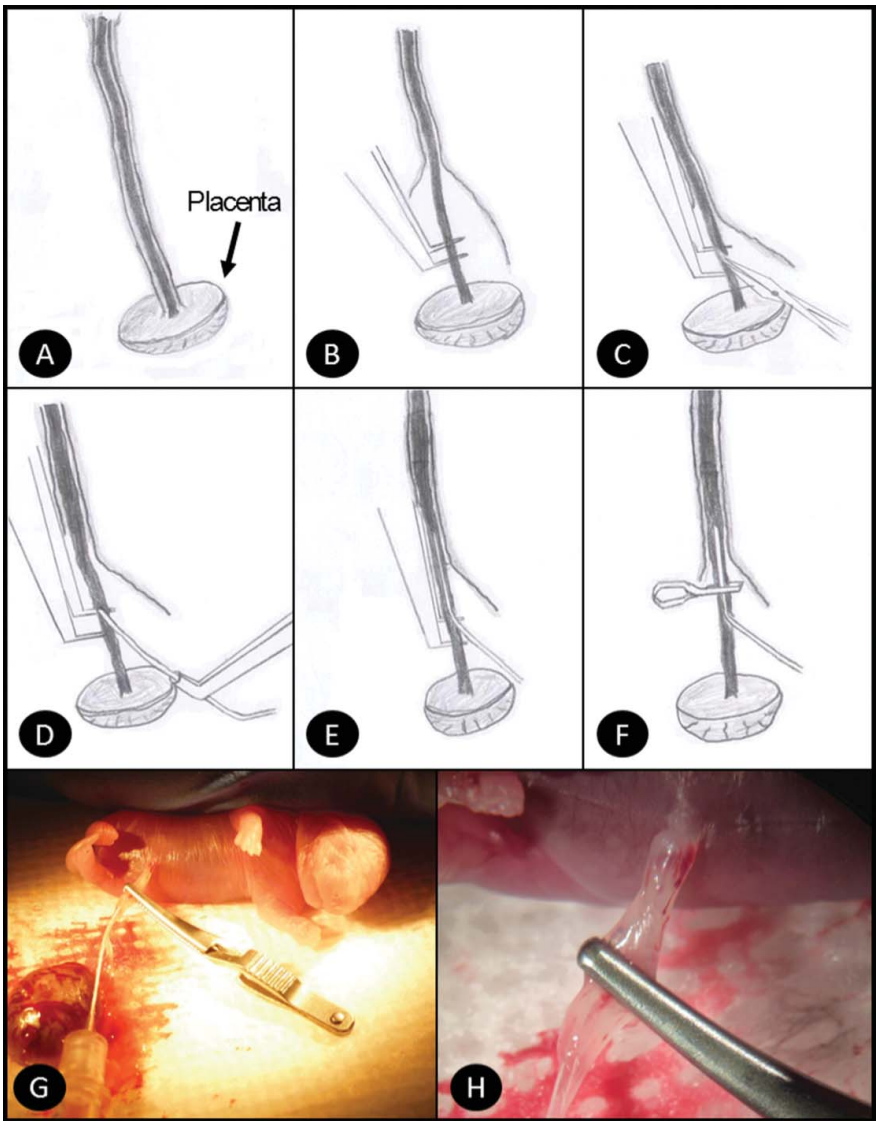

FIGURE 1 Technique of cannulation of the umbilical vein (UV) of newborn rat pups. (A) The placenta (black arrow) is placed on a gauze pad and the umbilical cord straightened for exposure; (B) The membrane covering the UV and arteries is dissected and the vein is separated from the arteries. A fine toothed forceps is placed beneath the exposed UV; (C) An oblique incision (~1.5 mm) is made in the UV that is then flushed with saline for visualization; (D) After slightly stretching the tip of PE-10 tubing to make it thinner, the tube is held with vessel cannulation forceps at an angle of 30å to the UV and the vein cannulated; (E) The tube is gently advanced into the vein for ~1.5 cm; (F) The tube is fixed in the UV with an atraumatic vessel clip; (G)(H) Images showing the completed cannulation.

Vimentin Staining of MSC in the Mucosa of Intestinal Villi

Frozen sections of OCT-embedded intestines were prepared and sections were rinsed in PBS three times, incubated with mouse antiVimentin monoclonal antibody (Thermo Scientific; IL, USA; http://thermoscientific.com/ab) overnight at 4°C, rinsed with PBS three times again, and incubated with Cy3 labeled donkey anti-mouse antibody for 1 hr at room temperature. Fluorescence was observed under a fluorescent microscope (Axioscope, Carl Zeiss; Jena, Germany) using GFP and Cy3 channels at 400× magnification.

RESULTS

Multipotent Properties of Mouse BM-MSC

The SC characteristics of mouse BM-MSC were verified in vitro by their ability to differentiate into osteocytes and adipocytes in the presence of specific

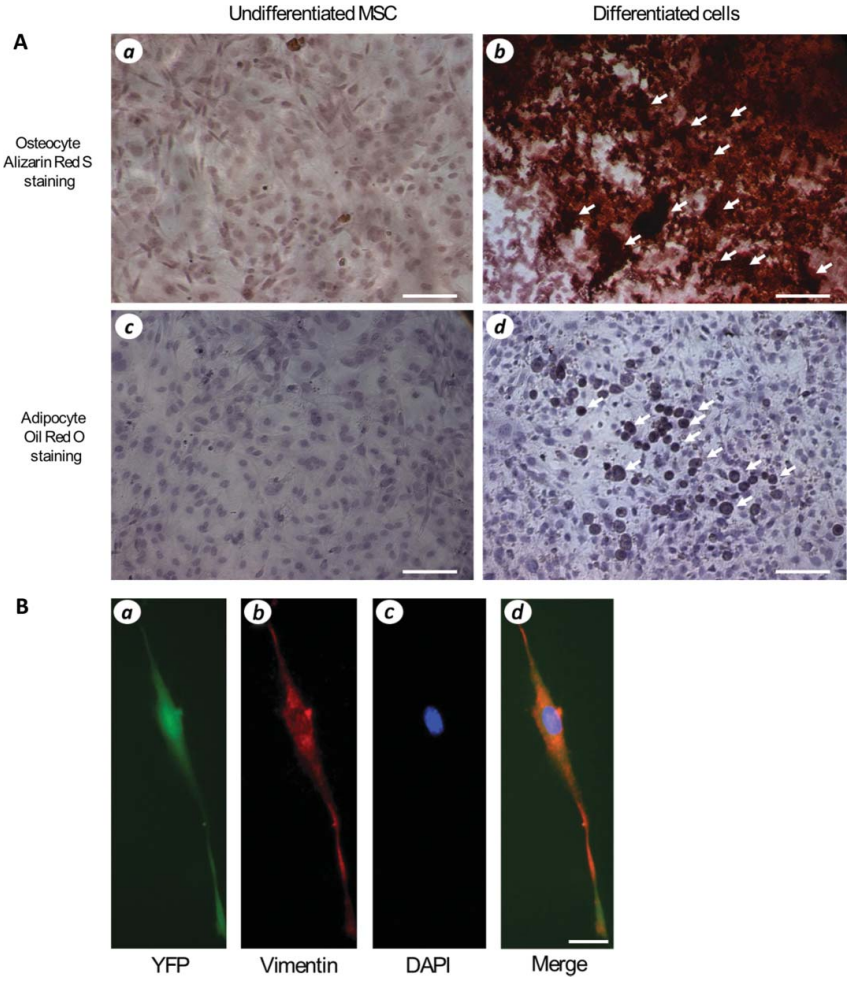

FIGURE 2 In vitro MSC differentiation assay. (A) MSC differentiation. (*a*) and (*c*) undifferentiated MSC; (*b*) differentiated MSC grown in osteogenesis medium had extracellular calcium deposits and stained positively with Alizarin Red S (white arrows); (*d*) differentiated MSC grown in adipogenesis medium had accumulation of lipid droplets and stained positively with Oil Red O (white arrows). 100× magnification, scale bar = 50 μm. (B) MSC immunocytochemistry. (*a*) YFP, green fluorescence; (*b*) vimentin, red fluorescence; (*c*) DAPI nuclear staining; (*d*) merged images. 400× magnification, scale bar = 15 μm. Abbreviations: MSC, mesenchymal stem cells; YFP, yellow fluorescent protein; DAPI, 4′,6-Diamidino-2-Phenylindole Dihydrochloride.

induction media. MSC were able to differentiate into both osteocytes and adipocytes (Figure 2A). Osteogenic differentiation was associated with extracellular precipitate stained with alizarin Red S corresponding to calcium deposits (Figure 2A, panel *b*). Adipogenic differentiation was accompanied by the accumulation of lipid droplets stained by Oil-Red (Figure 2A, panel *d*). Undifferentiated control cells had no staining with either alizarin Red S or Oil-Red (Figure 2A, panels *a* and *c*).

Vimentin Staining of YFP-labeled MSC

Cultured MSC had a spindle-like shape. As expected, all MSC had YFP expression. Vimentin-positive cells had red cytoplasmic staining (Figure 2B). All MSC were positive for Vimentin expression.

Success of MSC Injection and Mean Operating Time

All UV injections were performed by the same operator (JY). The first three injections had an operating time of ~8 min each, and injection failed in two of the pups due to UV rupture. After the first three pups, the operating time decreased to 2.5–5.5 min per pup (mean operating time 3.9 min ± 1.1 min), and the injection success rate was 92.8% (77 out of 83).

Methylene Blue Dye Reached the Systemic Circulation after UV Injection

Upon injecting methylene blue dye, the dye entered the UV (Figure 3A and B). The skin of rat pups was pink

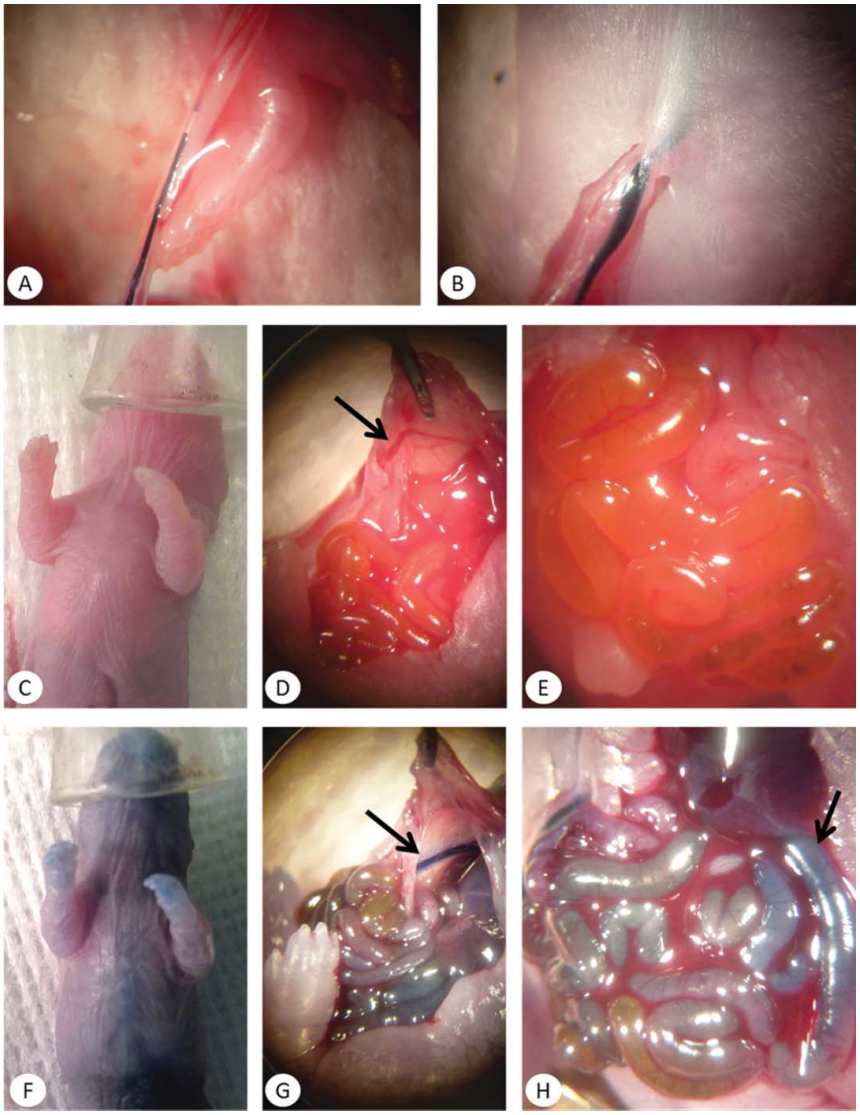

FIGURE 3 Tracking of methylene blue dye in the systemic circulation. (A) Prior to injection, showing the catheter in the umbilical vein (UV); (B) after injection, showing blue dye entering the UV; (C) pink skin prior to dye injection; (D) the internal UV (black arrow) prior to dye injection; (E) intestines prior to dye injection; (F) blue discoloration of the skin immediately after dye injection; (G) internal aspect of the UV (black arrow) after dye injection; (H) blue discoloration of the intestines several seconds after dye injection (black arrow).

prior to methylene blue injection (Figure 3C). Blue discoloration of the skin was noted immediately after injection in the order of chest, head, abdomen, and paws (Figure 3F). The internal aspect of the UV stained blue upon injection of the dye (Figure 3G). Bluish discoloration of the intestines was noted ~5 s after dye injection (Figure 3H).

Premature Newborn Rat Pups Have a Patent PFO and Pulse-Waves Change During and After MSC Injection

Doppler ultrasound imaging demonstrates a PFO with right-to-left shunting between the atria (Figure 4B). At the site of the PFO, pulse-wave ultrasound scanning showed baseline pulse-waves with right-to-left shunting prior to IV MSC injection (Figure 4C). During the injection of MSC, an extra wave was detected (Figure 4E), representing the extra blood flow through the PFO. After injection, the waves following the extra wave had a longer wavelength and higher wave peak (Figure 4F) compared to the waves at baseline (Figure 4D), indicating a higher speed of blood flow upon injection.

Mortality after IV MSC Administration in Adult Mice and Newborn Rat Pups

Immediately after IV MSC infusion, the mortality in adult mice was 21.7%, however, the mortality in premature rat pups was significantly decreased (6.43%,

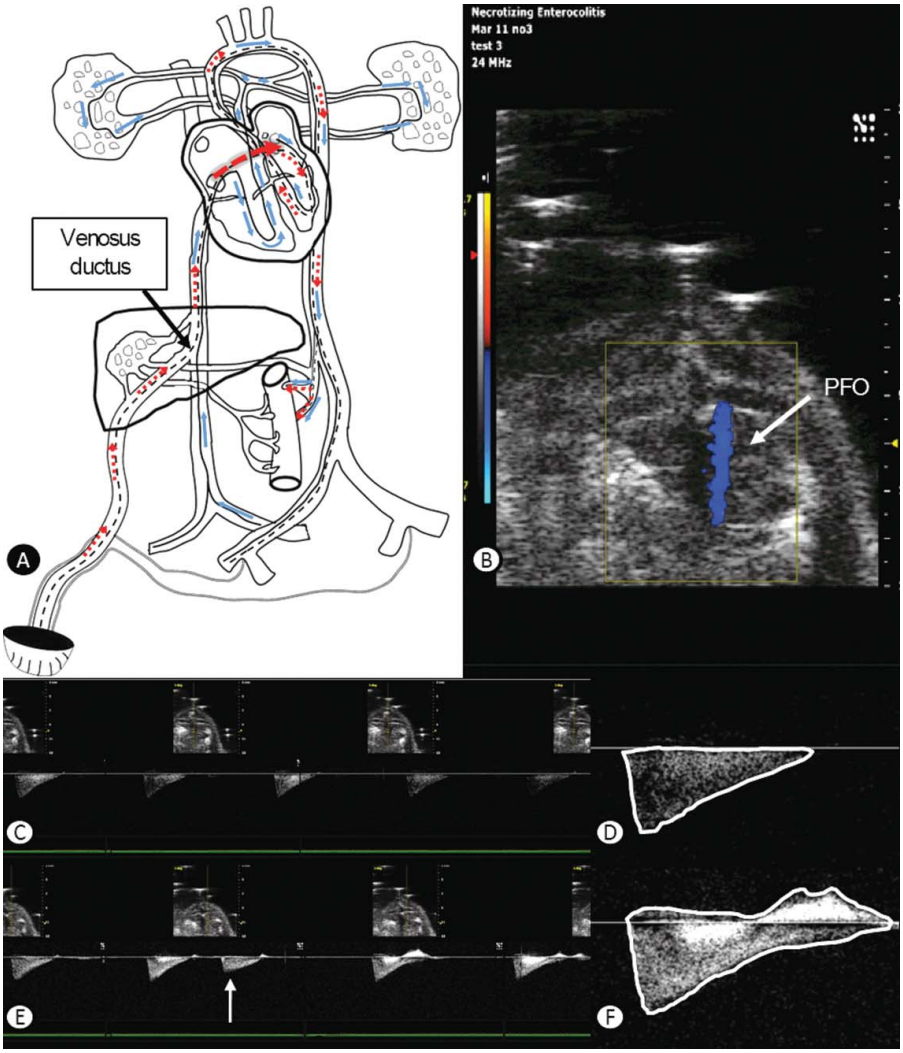

FIGURE 4 Right–to-left shunt in newborn premature rat pups. (A) Diagram illustrating a right-to-left shunt in a newborn rat pup. Small red dashed arrows show the route of the MSC delivered via the umbilical vein (UV) through the ductus venosus (black arrow) into the systemic circulation and the peripheral organs. Blue arrows show the blood circulation. The shunt is through the PFO from the right-to-left atrium (large red dashed arrow); (B) Doppler ultrasound imaging demonstrating PFO with right-to-left shunt between the atria (white arrow); (C) Pulse wave ultrasound scanning showing the right-to-left shunt prior to injection. The pulse waves are regular; (D) The wave shape of the shunt detected at the site of the PFO of a premature rat pup; (E) Extra wave detected after the normal wave at the time of injection of stem cells (white arrow); (F) Several following waves had a longer wavelength and higher wave peak. Abbreviations: MSC, mesenchymal stem cell; PFO, patent foramen ovale.

$p = .047$). Within 24 hr, the cumulative mortality in adult mice was 47.8%, whereas the cumulative mortality in rat pups was significantly less (23.4%, $p = .0352$). No control animals receiving saline injection died.

YFP-MSC Engraftment in Lungs, Heart and Intestines

As expected, negative control rat pups receiving saline injection only had no YFP positive MSC in the lungs, heart, or intestines (Figure 5A, panels *a*, *d*, *g*). YFP-MSC were identified in these organs after IV MSC administration (Figure 5A, panels *b*, *c*, *e*, *f*, *h*, *i*). Quantification of YFP-MSC engraftment revealed 15.8 ± 4.1 CPVF in the lungs, 2.9 ± 1.2 CPVF in the heart, and 19.8 ± 5.0 CPVF in the intestines (Figure 5B).

YFP-MSC Vimentin Expression in Intestinal Mucosa

YFP positive MSC were noted in the mucosal layer of the villi (Figure 5C). Vimentin expression co-localized with YFP expression in MSC.

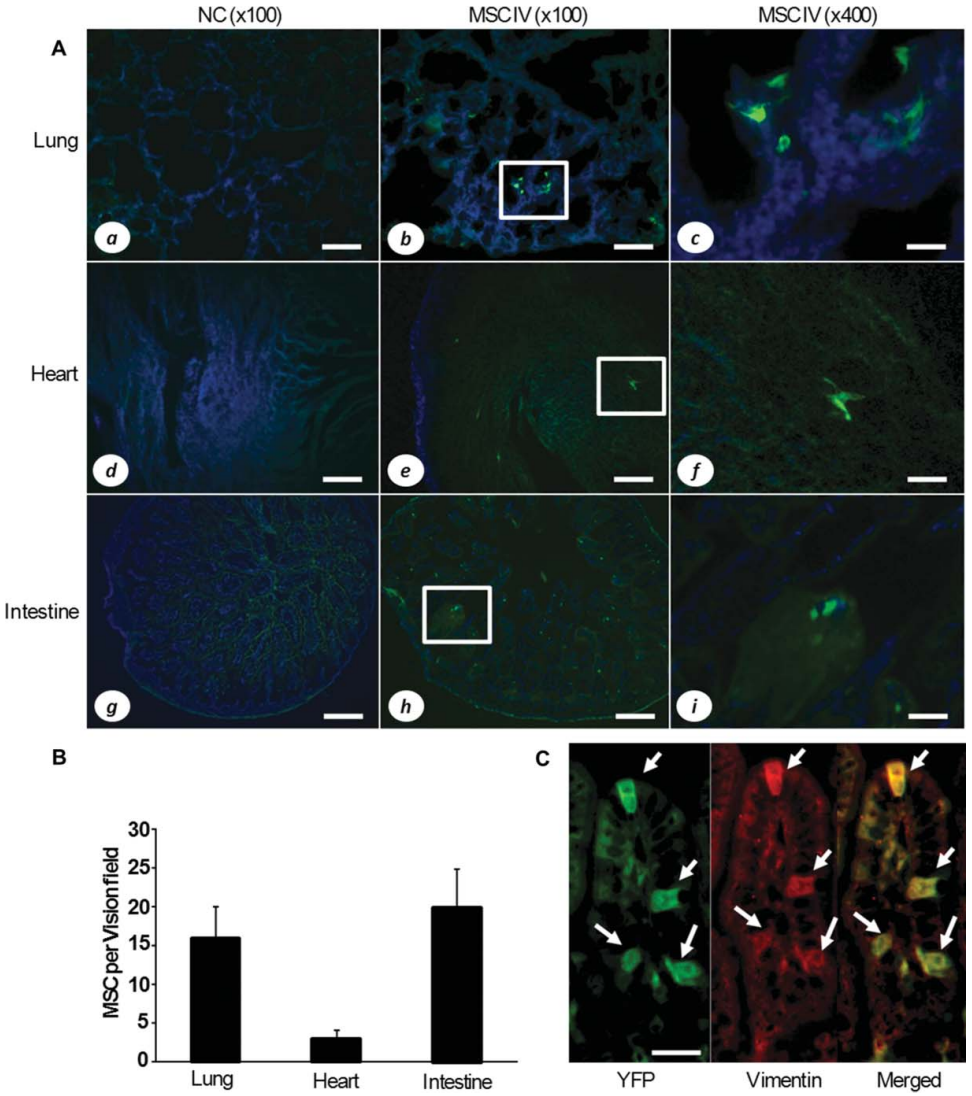

FIGURE 5 Distribution, quantification, and identification of MSC after systemic infusion. (A) Distribution of MSC in the lung, heart, and intestine after IV injection in newborn rat pups. Nuclei are stained with DAPI. YFP positive cells are demonstrated by green fluorescence. (*a*), (*d*), (*g*) Control pups that received saline injection only, 100× magnification. Scale bar = 50 μm; (*b*), (*e*), (*h*) Pups received 300 × 10^3 MSC IV, 100× magnification. Scale bar = 50 μm; (*c*), (*f*), (*i*) High power view of the areas contained within the white rectangles in panels *b*, *e*, *h*. 400× magnification. Scale bar = 12.5 μm; (B) Quantification of YPF positive cells in the lung, heart, and intestine; (C) Immunofluorescence of vimentin in the cytoplasm of MSC in the mucosa of the intestine 96 hr after systemic MSC administration. Scale bar = 12.5 μm. Abbreviations: MSC, mesenchymal stem cell; DAPI, 4′,6-Diamidino-2-Phenylindole Dihydrochloride; YFP, yellow fluorescent protein.

DISCUSSION

For MSC therapy to be broadly applied as a clinical therapeutic strategy in newborns, a simple IV approach for MSC biodistribution is desirable. Two decades ago, a technique for UV injection was first developed in rat pups delivered prematurely on days 18 and 20 of gestation, with drugs injected through the UV found in the systemic circulation [20]. However, the pups could not be kept alive after injection because the umbilical arteries were cut. We modified this technique so that after MSC injection we can keep prematurely delivered newborn pups alive. Cannulation using a fine, clear, and soft plastic tube in the UV allows us to judge whether or not the injection is successful. One person alone can perform the injection, with the average operating time of less than 4 min and a success rate of 92.8%.

The injection of methylene blue through the UV confirmed dye delivery to the chests, brain, limbs, and intestines immediately after injection. In order to track the fate of MSC after systemic administration, we quantified the number of YFP-positive cells that engrafted in different organs 96 hr after injection. The intestines had the highest numbers of engrafted YFP-MSC followed

by the lungs and then the heart. The MSC marker vimentin was expressed in YFP-MSC in the mucosa of the intestines 96 hr after MSC administration, confirming MSC engraftment.

In premature rat pups, the ductus venosus connecting the UV and the inferior vena cava is not tightly closed immediately after birth [21], providing a period of time during which IV MSC injection through the UV into the systemic circulation can be performed. Compared to MSC injection in adult mice, we found that prematurely delivered newborn rat pups had a significantly lower death rate immediately after MSC injection and 24 hr after injection. We speculate that this difference in mortality is due to the different anatomy in adults and neonates, specifically the presence of a right-to-left shunt in the circulation of newborn rat pups. Rat pups born on gestational day 21 are equivalent to human fetuses at 34 weeks of gestation [22]. There are three major shunts present at this time, which permit much of the blood returning to the right side of the heart to continue directly into the systemic circulation, thus bypassing the pulmonary circulation. The three shunts are the PFO, the ductus arteriosus and the interventricular shunt, of which the PFO is the most important physiological right-to-left shunt in prematurely delivered rat pups. MSC injected into the UV pass through the ductus venosus to the IVC, and then from the right atrium to the left atrium through the PFO. We confirmed the existence of a PFO in our rat pups using ultrasound. The right-to-left shunt through the PFO allows a portion of the injected MSC suspension to bypass the pulmonary circulation and go directly into the systemic circulation and subsequently to the peripheral organs. We believe that a PFO improves survival after MSC UV injection in newborn rat pups by decreasing MSC entrapment in the lungs.

In the current studies, one of our goals was to compare the effects of MSC injection in newborn and adult animals. We compared UV injection in rat pups and tail vein injection in adult mice for the following reasons: (1) UV and tail vein injections are both IV systemic routes of administration. Systemic administration is likely to be an effective and clinically applicable route of administration in patients. (2) We used adult mice instead of adult rats for comparison to rat pups in order to more closely approximate animal weights. An adult rat is $\sim$ 50–60 times heavier than a premature newborn rat pup (250–300 grams vs. 5 g), whereas an adult mouse is only approximately five times heavier than a newborn rat pup (25 grams vs. 5 g). (3) Adult mice and adult rats have very similar anatomic structure of the heart and lungs. In separate studies, we have been using an adult animal model of intestinal ischemia/reperfusion injury [23, 24], and are now examining the effects of MSC infusion in this model. Based on our current findings, we speculate that adult animals may be more vulnerable to the pulmonary complications of IV MSC infusion compared to newborn animals.

Previous studies from other laboratories have focused on strategies of cell delivery in large animals including nonhuman primates and pigs. A recent study showed the feasibility of recurrent islet cell delivery through a vascular access port with the catheter tip located in the splenic vein [25]. Since we are interested in cell delivery to neonates, the small size of the vascular system creates considerable challenges for IV SC administration. The ability to successfully perform neonatal rat IV injections should prove useful in studies of many neonatal diseases associated with premature delivery. For the first time, we now describe an innovative and nontraumatic technique for systemic MSC administration in premature rat pups. This technique can be used in animal models of newborn disease including experimental necrotizng enterocolitis, the most common gastrointestinal emergency in premature infants [26]. We utilize an experimental rat model of NEC that uses premature newborn rat pups as the experimental animals [27–30]. In our NEC model, enteral administration of heparin-binding EGF-like growth factor (HB-EGF) can decrease the incidence of NEC by $\sim$50%. Preliminary studies show that we can significantly decrease the incidence of NEC further with the administration of HB-EGF in conjunction with MSC administered using the technique described here (unpublished observations).

Taken together, we present evidence that IV MSC infusion through the UV is a convenient, safe, and effective method of systemic MSC administration. The patent ductus venosus allows MSC to enter the systemic circulation, and the physiological right-to-left shunt in the prematurely delivered rat allows a portion of the injected MSC to bypass the lungs, thereby decreasing the risk of pulmonary entrapment. This allows increased numbers of MSC to engraft into peripheral organs rather than being trapped in the lung, thus decreasing the pulmonary first-pass effect [16, 31]. MSC can be successfully delivered to the heart, lung, and intestine of newborn pups using this method. This technique will be beneficial in examining the effects of MSC transplantation in animal models simulating neonatal diseases.

CONCLUSIONS

We demonstrate here for the first time a nontraumatic technique for systemic administration of SCs via the UV to newborn rat pups. The technique is easy to perform, has a short operating time, a high success rate, a high efficiency of systemic MSC delivery, and a low mortality rate. We show that the delivered MSC enter the systemic circulation through the ductus venosus, pass the pulmonary barrier through the right-to-left

shunt after injection, and engraft in peripheral organs including the lungs, heart, and intestines. Successful administration of MSC via the UV may illustrate a novel route of administration for newborns requiring MSC transplantation in the future.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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