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Exosomes Secreted from Bone Marrow-Derived Mesenchymal Stem Cells Protect the Intestines from Experimental Necrotizing Enterocolitis

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Abstract

Purpose—Treatment options for necrotizing enterocolitis (NEC) remain inadequate. Bone marrow-derived mesenchymal stem cells (BM-MSCs) can protect the intestines from NEC. Exosomes are nanoparticle-sized vesicles with important cell signaling capabilities. The objective of this study was to determine whether BM-MSC-derived exosomes can prevent NEC.

Methods—Rat pups were either breast fed (Group 1) or were subjected to experimental NEC and randomized to receive either no treatment (Group 2) or an intraperitoneal (IP) injection of PBS (Group 3), BM-MSC (Group 4), or BM-MSC-derived exosomes (Group 5). Histologic injury grade and intestinal permeability were determined. The effect of BM-MSC-derived exosomes on IEC-6 intestinal epithelial cells in an *in vitro* scrape model of wound healing was also determined.

Results—Animals exposed to NEC that were either untreated or that received PBS alone had a NEC incidence of 46% and 41%, respectively (p=0.61). Compared to untreated pups, the incidence of NEC was significantly lower in pups treated with either BM-MSC (9%, p=0.0003) or MB-MSC-derived exosomes (13%, p=0.0008). Similar results were found for intestinal permeability. Wound healing in IEC-6 cells was significantly increased by BM-MSC-derived exosomes.

Conclusion—BM-MSC-derived exosomes protect the intestines from NEC and may represent a novel, cell-free, preventative therapy for NEC in the future.

Keywords

Mesenchymal stem cells; exosomes; necrotizing enterocolitis; NEC

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1. INTRODUCTION

Necrotizing enterocolitis (NEC), the most common surgical emergency in neonates, has an incidence of 1–3 cases per 1000 live births in the United States, affecting 2000–4000 predominantly premature newborns [1], [2]. Severe NEC comprises 27% to 63% of all cases, often requiring substantial surgical resection of nonviable intestine [2]. NEC mortality ranges from 20% to 50%, resulting in approximately 1000 deaths each year [2]. NEC is the leading cause of short bowel syndrome (SBS) in the pediatric population. Although significant research has been performed to identify novel preventive and therapeutic interventions, the incidence, morbidity, and mortality from NEC remains unacceptably high.

Research on mesenchymal stem cell (MSC) therapy for injured and diseased tissues, including the intestines, has been promising [3], [4]. MSCs have the ability to engraft into injured tissues and promote healing through paracrine mechanisms [5], [6], [7], [8], [9]. Recent studies have shown that MSCs administered either intraperitoneally (IP) or intravenously (IV) engraft into injured intestinal tissue and decrease the incidence of experimental NEC in rats [10], [11]. However, the number of MSC that engraft into injured tissues may not be sufficient to account for their robust overall protective effects. This suggests that additional mechanism(s) mediate these protective effects, including secreted bioactive factors that confer protection in a paracrine fashion [12], [13], [14].

Exosomes are nanovesicles produced by the endosomal pathway that are initially contained intracellularly within multivesicular bodies. They are secreted into the extracellular space when these multivesicular bodies fuse with the cell membrane [15]. Once thought to contain cellular material no longer valuable to the cell, research over the last decade has revealed that exosomes are important in cellular communication and signal transduction [16], [17], [18]. Exosomes secreted by many different types of cells affect cell signaling by interacting with receptors on target cell membranes, modifying the extracellular milieu surrounding target calls, or by fusing with the target cell membrane and releasing their contents into the target cell cytoplasm [19]. The contents of exosomes include micro-, messenger-, and small interfering RNAs, as well as proteins such as growth factors [20], [21], [22], [23]. The molecular cargo of exosomes is variable depending upon the nature of the secreting cell. Exosomes secreted by MSCs have been shown to contain anti-apoptotic miRNAs, to promote epithelial and endothelial wound healing and angiogenesis, and to contain growth factor receptor mRNAs known to promote wound healing [24] [25], [26], [27], [28], [29]. The goal of this study was to determine if exosomes secreted from BM-MSCs could protect the intestines from experimental NEC.

2. MATERIALS AND METHODS

2.1 Ethics statement

All animal procedures (Protocol #04203AR) were approved by the Institutional Animal Care and Use Committee (IACUC) of the Research Institute at Nationwide Children’s Hospital.

2.2 Isolation and identification of BM-MSCs

These studies utilized a murine bone marrow-derived MSC line that was previously established and characterized [30]. Murine bone marrow-derived MSC harvested from H2K-GFP transgenic mice were previously subjected to flow cytometry to confirm lack of expression of CD45 and other hematopoietic lineage markers (TER119, CD3, B220, CD11b, and Gr-1) and expression of the MSC markers CD29, CD49e, CD90, CD105, and Sca-1. The trilineage (osteogenic, adipogenic, and chondrogenic) differentiation potential of these cells was previously confirmed.

2.3 Preparation of BM-MSCs for in-vivo administration

Adherent BM-MSCs were grown in D-MEM/F12/Glutamax-I™ (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) in T-75 flasks until 80% confluent. Cells were then trypsinized (0.25% trypsin, Gibco Invitrogen, Carlsbad, CA) for 3 min, followed by trypsin neutralization using media containing 10% FBS. Cells were quantified using a hemacytometer, centrifuged at 180xg for 5 min at 4°C, and resuspended in sterile phosphate buffered saline (PBS) at a concentration of $3x10^5$ cells per 50 μl. This preparation of cells was then loaded into 0.3 ml low-dose U-100 insulin syringes with 29 gauge needles (Becton Dickinson, Franklin Lakes, NJ) immediately prior to IP injection.

2.4 Isolation and characterization of exosomes

BM-MSCs were cultured until ~80% confluent, and were then cultured in serum-free media (SFM) for 48h, at which time exosomes were isolated from the BM-MSC-conditioned media (CM) by one of two methods. The first method, used for *in vivo* studies, was performed according to the manufacturer's instructions for the P100 PureExo Exosome Isolation kit (101bio, Palo Alto, CA). Exosomes were isolated from one T-75 flask containing ~80% confluent BM-MSCs, resuspended in 150 μl PBS, and used for three separate IP injections (50 μl/pup). These isolated exosomes were stored at 4°C and used within 72 hours or were frozen at −80°C. Exosomes isolated using this method were characterized by nanoparticle tracking analysis (NTA) using the NanoSight NS300 (Malvern Instruments Inc., Westborough, MA). Quantification indicated that this isolation method yielded ~$2.5x10^9$ BM-MSC-derived exosomes / 50 μl volume. The second method, used for *in vitro* wound healing studies, used serial centrifugation to obtain both BM-MSC-derived exosomes as well as exosome-depleted BM-MSC-CM for use as a control [31], [32]. One T-75 flask containing ~80% confluent BM-MSCs was used to prepare BM-MSC-CM which was collected and centrifuged at 300xg for 10 min to pellet cells which were discarded. The supernatant was collected and centrifuged at 2,000xg for 20 min, and the resulting supernatant was centrifuged at 10,000xg for 30 min to pellet contaminating cellular debris, which was discarded. The remaining supernatant was then ultra-centrifuged at 100,000xg for 18h to obtain both isolated exosomes (pellet) as well as exosome-depleted BM-MSC-CM (supernatant) [32]. The pellet was resuspended in 150 μl PBS, and both isolated exosomes and exosome-depleted BM-MSC-CM were stored at 4°C and used within 3 days or frozen at −80°C. These preparations were used for three separate treatments of IEC-6 cells, at a volume of 50 μl each, as described in section 2.9. Exosomes isolated using both methods

showed strong Western blot immunostaining to the well-established exosome markers CD-9 and flotillin-1.

2.5 Animal model of NEC

Premature Sprague-Dawley rat pups were delivered by caesarean section under CO_2 anesthesia at E-21 of gestation. Briefly, pups were then subjected to a modification of the experimental NEC protocol initially described by Barlow *et al.* [33]. Pups were kept in an incubator at 35°C with 50% humidity and fed by orogastric gavage five times daily using a NICC-NATE® 1.9 French single lumen silicone catheter (Utah Medical Products Inc., Midvale, Utah). The hypertonic formula used for feedings [15 g of Similac 60/40 (Ross Pediatrics, Columbus, Ohio) in 75 ml of Esbilac (Pet-Ag, New Hampshire, IL] provided 836.8 kJ/kg per day. Feeds were started at 0.1 ml of formula per feed with the volume of formula advanced each day by 0.1 ml per feed to a maximum of 0.4 ml per feed by the fifth day of life. Immediately after feeding, pups were exposed twice daily to hypoxia (100% nitrogen for 90 sec), followed by hypothermia (4°C for 10 min) until the end of the experiment at 96h of life.

2.6 In Vivo Experimental Design

Newborn rat pups were randomized to one of the following groups: Group 1: breast-fed control pups (N=8) that were placed with a surrogate dam so that they could receive physiologic breast milk feeds; Group 2: NEC only (N=46); Group 3: NEC plus a single IP dose of 50 μl PBS vehicle (N=59); Group 4: NEC plus BM-MSCs in 50 μl PBS IP (N=35); or Group 5: NEC plus BM-MSC-derived exosomes in 50 μl PBS IP (N=40). Pups were exposed to BM-MSC or BM-MSC-derived exosomes 5h after delivery. Pups exposed to NEC were sacrificed by decapitation upon the development of clinical signs of NEC. All pups surviving for 96h of life were sacrificed at that time. After each sacrifice, the small intestine was immediately removed and formalin-fixed for histologic analysis.

2.7 Histologic evaluation of experimental NEC injury

The distal small intestine of each pup was removed upon sacrifice and fixed in 10% formalin for 24h. The fixed tissue was paraffin-embedded and transverse sections were prepared and stained with hematoxylin and eosin (H&E). H&E stained sections were graded by two independent observers using a standard histological injury scoring system [34] as follows: Grade 0, no villus damage; Grade 1, distal villus enterocyte detachment; Grade 2, sloughing of enterocytes to the mid villus level; Grade 3, loss of the entire villus with preservation of the crypts; and Grade 4, transmural necrosis. Injury grades of 2, 3, or 4 were defined as NEC, while injury grades 3 and 4 were defined as severe NEC.

2.8 Evaluation of intestinal mucosa permeability

Fluorescein isothiocyanate (FITC) labeled dextran (FD70, molecular weight 73,000) (Sigma-Aldrich Inc., St. Louis, MO) was used to assess mucosal permeability as described previously [35]. Briefly, rat pups from each experimental group received 750 mg/kg FD70 suspended in sterile PBS by orogastric gavage 46h after C-section. Four hours later, pups were sacrificed and serum levels of FD70 were measured by spectrophotofluorometry. The

FD70 concentration in the plasma of each pup was calculated from a standard curve of known FD70 concentrations.

2.9 Intestinal epithelial cell scrape wound healing assay

Scrape wounding was performed as previously described [36], [37]. IEC-6 cells were seeded to each well of a 12 well plate at 80% confluence and incubated in DMEM/F12/Glutamax-I™ (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% FBS at 37°C in 5% CO_2. Once a monolayer formed, cells were serum starved overnight. Two perpendicular, intersecting lines were drawn across the bottom of each well, separating each well into quadrants. A sterile 200 μl pipette tip was used to create two linear scrape wounds in the monolayer, parallel to each of the marked lines, and intersecting in the top right quadrant. Cells were then washed three times with Hank's balanced salt solution (HBSS) and 1.0mL of one of the following was added: (1) SFM alone (control), (2) SFM containing 50 μl PBS, (3) SFM containing 50 μl BM-MSC-derived exosomes, (4) BM-MSC-CM, or (5) exosome-depleted BM-MSC-CM. Photomicrographs of the horizontal scrape immediately to the right of the intersection of the vertical and horizontal scrapes were taken immediately after wounding (0h) and eight hours after wounding (8h) using an inverted phase contrast light microscope (Carl Zeiss Inc, Thornwood, NY). Each experiment was performed five times (N=5) in triplicate. The width of the wounds in each photomicrograph was measured using Image J software (National Institutes of Health, Bethesda, MD). The percent healing for each wound was calculated as: (W8h) / (W0h) x 100, where W8h and W0h represent the average wound widths at 8h and 0h, respectively. The average percent healing of each wound was normalized to the average percent healing of control (SFM alone) wounds, and reported as fold change of control.

2.10 Statistical analyses

All values represent mean ± SEM. The Pearson Chi-square test was used to assess for statistical significance between the incidence of NEC and of severe NEC. Statistical significance between serum levels of FITC-dextran was assessed using one-way ANOVA followed by Students t-test for pairwise comparison. Statistical significance between IEC-6 cell scrape healing rates were determined using Mann-Whitney U test. P values of < 0.05 were considered to be statistically significant.

3. RESULTS

3.1 Exosomes derived from BM-MSCs decrease the incidence and severity of experimental NEC in premature newborn rats

Representative histologic NEC injury grades are shown in Figure 1A. The overall incidence of NEC (Grades 2, 3 or 4) is shown in Figure 1B. Breast fed pups had no intestinal histologic injury. Pups exposed to NEC alone had a NEC incidence of 46% and pups exposed to NEC+PBS had a NEC incidence of 41% (p=0.609). However, pups exposed to NEC+BM-MSCs had a NEC incidence of 9% and pups exposed to NEC+BM-MSC-derived exosomes had a NEC incidence of 13% (p=0.0003 and p=0.0008 respectively, compared to pups exposed to NEC). Differences in the incidence of NEC were not significant between pups receiving NEC+BM-MSC and pups receiving NEC+BM-MSC-derived exosomes

(p=0.582). The incidence of severe NEC (Grades 3 or 4) is shown in Figure 1C. Pups exposed to NEC alone had a severe NEC incidence of 28% and pups exposed to NEC+PBS had a severe NEC incidence of 24% (p=0.598). However, pups exposed to NEC+BM-MSCs had a severe NEC incidence of 9% and pups exposed to NEC+BM-MSC-derived exosomes had a severe NEC incidence of 10% (p=0.027 and p=0.034 respectively, compared to pups exposed to NEC). Differences in the incidence of severe NEC were not significant between pups exposed to NEC+BM-MSC and pups exposed to NEC+BM-MSC-derived exosomes (p=0.832).

3.2 Exosomes derived from BM-MSCs preserve gut barrier function after experimental NEC

In breast fed pups, the serum levels of FD70 were extremely low (0.55 ± 0.04 μg/ml) (Figure 2). Pups exposed to NEC alone had serum levels of FD70 that were significantly higher than those of the breast fed pups (20.32 ± 3.43 μg/ml, p=0.0001, indicative of increased intestinal permeability and decreased gut barrier function. Compared to pups exposed to NEC alone, pups exposed to NEC+PBS had similarly elevated serum levels of FD70 (17.76 ± 3.95 μg/ml, p=0.626). However, pups exposed to NEC+BM-MSC and pups exposed to NEC +BM-MSC-derived exosomes both had significantly lower serum levels of FD70 compared to pups exposed to NEC (9.65 ± 2.03 μg/ml, p=0.013 and 9.38 ± 2.34 μg/ml, p=0.015, respectively). There was no statistically significant difference in serum levels of FD70 between pups exposed to NEC+BM-MSC and pups exposed to NEC+BM-MSC-derived exosomes (p=0.930).

3.3 Exosomes derived from BM-MSCs promote wound healing of IEC-6 cells in vitro

There was no significant difference in wound healing observed between IEC-6 cells exposed to serum-free media (SFM) compared to those exposed to SFM+PBS (1 *vs.* 0.986, p=0.656) (Figure 3). However, significantly improved healing rates were observed in cells exposed to both SFM+BM-MSC-derived exosomes (1.71, p=0.012) and SFM+BM-MSC CM (1.38, p=0.0119), compared to cells exposed to SFM+PBS. Conversely, in cells that were exposed to BM-MSC CM that had been depleted of exosomes, no significant difference in wound healing was seen compared to cells exposed to SFM+PBS (1.07, p=0.210).

4. DISCUSSION

We have previously shown that MSC can prevent experimental NEC when administered either IP or IV [11]. MSCs have been shown to engraft into injured intestinal tissues, but the number of MSCs that engraft is generally not sufficient to account for the robust overall protective effects observed, suggesting that additional mechanism(s) exists [12], [13], [14]. This is supported by research describing the effects of paracrine signaling of MSCs in models of experimental injury [7], [8], [9].

Exosomes have emerged over the last decade as important mediators of cell signaling, and are implicated in cellular growth and healing in addition to many other cellular functions [16], [17], [18], [24], [26]. Exosomes are attractive candidates for mediators of paracrine MSC signaling due to the nature of their cargo, which includes micro-, messenger-, and

small interfering RNAs, as well as proteins such as growth factors [15], [19], [20], [21], [22], [23]. Exosomes secreted by MSCs have been shown to promote epithelial and endothelial wound healing and angiogenesis, and to contain anti-apoptotic miRNAs and growth factor receptor mRNAs associated with wound healing [25], [27], [28], [29].

Our current findings demonstrate that IP injection of exosomes isolated from BM-MSCs can reduce the incidence and severity of experimental NEC, and can preserve the integrity of the gut barrier, in a neonatal rat model of NEC. When comparing the effects of administering BM-MSC-derived exosomes to the effects of administering BM-MSCs themselves, there were no statistically significant differences in the effects on NEC incidence, NEC severity, or gut permeability. This demonstrates that BM-MSC-derived exosomes can independently protect the intestines from experimental NEC, suggesting that exosomes are the main paracrine effectors of these cells, and may be predominantly responsible for the ability of BM-MSCs to protect the intestines from NEC.

Additionally, we show that BM-MSC-derived exosomes increase the rate of intestinal epithelial cell (IEC) wound healing in an *in vitro* model of IEC scrape wounding. We compared the effects of SFM alone, isolated BM-MSC-derived exosomes, BM-MSC-CM, and BM-MSC-CM that had been depleted of exosomes. Compared to SFM alone, exposure to either SFM+exosomes, or to SFM+CM, increased the rate of IEC scrape wound healing. Exposure of IEC cells to SFM+exosomes resulted in the most robust increase in wound healing. Importantly, the ability of BM-MSC-CM to increase IEC wound healing was nearly completely abrogated when it was depleted of exosomes. This further supports our conclusion that exosomes play a predominant role in the paracrine signaling of BM-MSCs.

There are a many unanswered questions regarding the role of exosomes in protecting the intestines from NEC, which will be addressed in future experiments. Our current study employed IP administration of BM-MSC and BM-MSC-derived exosomes. We have previously shown that IV administration of BM-MSC leads to increased MSC engraftment into NEC-injured intestine [11]. Future studies will compare IV and IP administration of exosomes in our experimental NEC model. In addition, the current studies exclusively examined exosomes derived from BM-MSC. We have previously shown that neural stem cells (NSC) can also protect the intestines from NEC [38]. Future studies will examine the efficacy of exosomes derived from different types of stem cells, including NSC and amniotic fluid (AF)-derived MSC. Clinically, whereas harvesting BM-MSC would require bone marrow aspiration, AF can easily be obtained at the time of delivery and MSC harvested from the fluid. We have previously shown that AF-MSC can protect the intestines from intestinal injury [39]; therefore exosomes from AF-MSC may hold therapeutic potential for NEC. Furthermore, we have previously shown that administration of heparin-binding EGF-like growth factor (HB-EGF) protects the intestines from experimental NEC, and that the magnitude of protection was increased when HB-EGF was co-administered with BM-MSCs. Future experiments will be performed to determine whether the protection conferred by BM-MSC-derived exosomes can be augmented by loading the exosomes with HB-EGF, thus using exosomes as delivery vehicles for HB-EGF. Finally, studies are planned to identify the key agent(s) contained in the exosome cargo responsible for the protective effects of exosomes against NEC.

5. CONCLUSIONS

In summary, our current findings demonstrate that stem cell-derived exosomes are as effective as stem cells themselves in protecting the intestines from NEC. This is an important finding as it supports a potential future cell-free therapy for the treatment of this disease.

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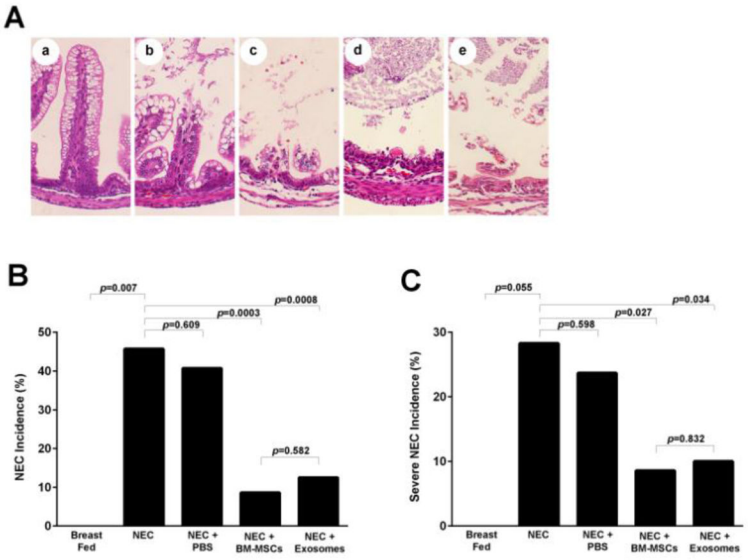

Figure 1. Effect of BM-MSC and BM-MSC-derived exosomes on the incidence and severity of NEC

(A) Histological grading of intestinal injury from neonatal rats. Shown are representative images of the following injury grades: (a) Grade 0; (b) Grade 1; (c) Grade 2; (d) Grade 3; and (e) Grade 4. Magnification 200x. (B) Incidence of NEC (Grades 2, 3 or 4 injury). (C) Incidence of severe NEC (Grade 3 or 4 injury only). The numbers of animals used to derive the data shown in B and C are as follows: BF (N=8), NEC (N=46), NEC+PBS (N=59), NEC +BM-MSCs (N=35), NEC+BM-MSC-derived exosomes (N=40). NEC, necrotizing enterocolitis; PBS, phosphate-buffered saline; BM-MSCs, bone marrow-derived mesenchymal stem cells.

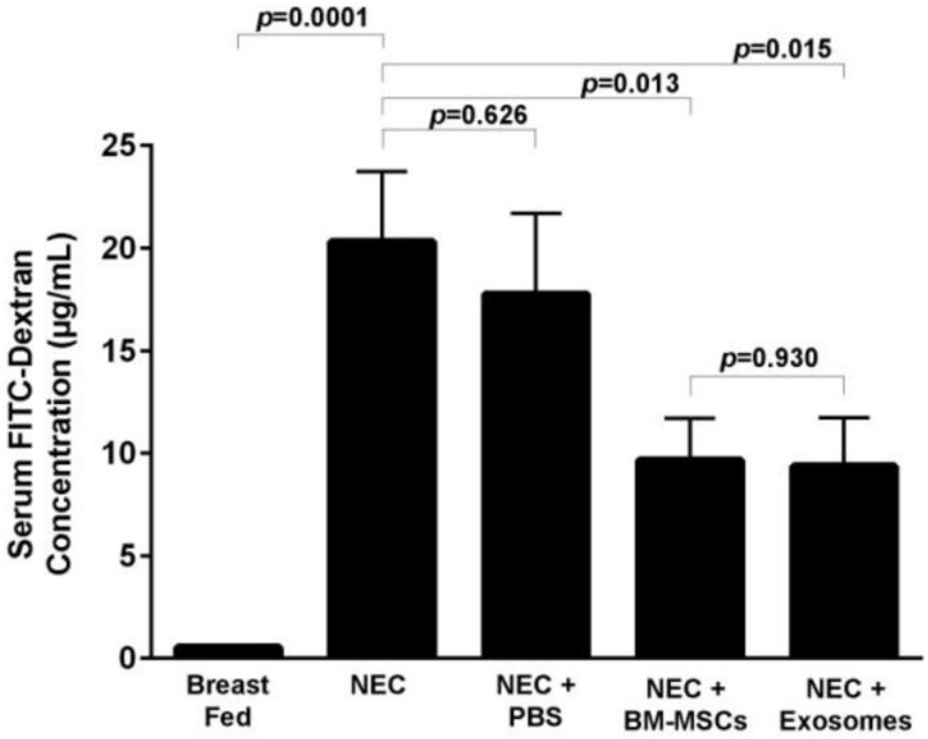

Figure 2. Effect of BM-MSC and BM-MSC-derived exosomes on intestinal permeability

The absorption of FITC labeled FD70 was used to quantify mucosal permeability. Pups received 750 mg/Kg FD70 by orogastric gavage 46h after C-section and 4h later pups were sacrificed and serum levels of FD70 measured. Higher concentrations of FD70 in the serum represent decreased gut barrier function. The numbers of animals in each group are as follows: BF (N=13), NEC (N=17), NEC+PBS (N=15), NEC+BM-MSCs (N=15), NEC +BM-MSC-derived exosomes (N=16).

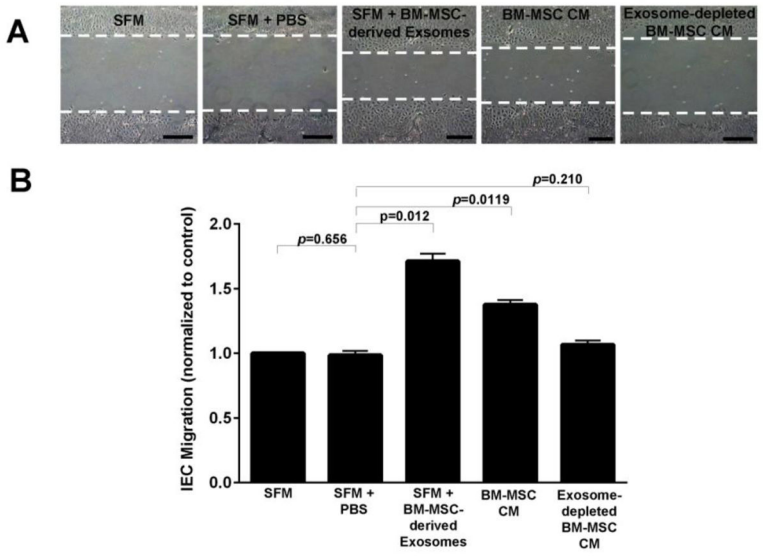

Figure 3. Scrape wound healing of IEC-6 cell monolayers *in vitro*

IEC-6 cell monolayers were serum starved, subjected to scraping, and 1.0 mL of one of the following was added: (1) SFM alone (control), (2) SFM containing 50 μl PBS, (3) SFM containing 50 μl BM-MSC-derived exosomes, (4) BM-MSC-CM, or (5) exosome-depleted BM-MSC-CM. (A) Appearance of scrape wounds with the indicated treatments 8 h after scraping. The dotted lines indicate the wound margins. (B) Quantification of scrape wound healing. Each experiment was performed four times in triplicate. The percent healing for each wound was calculated as: (W8h) / (W0h) x 100, where W8h and W0h represent the average wound widths at 8h and 0h, respectively. The average % healing of each wound was normalized to the average % healing of control wounds. SFM, serum-free media; PBS, phosphate-buffered saline; BM-MSCs, bone marrow-derived mesenchymal stem cells. Scale bar represents 200 μm.