Evaluating the efficacy of different types of stem cells in preserving gut barrier function in necrotizing enterocolitis

Christopher J. McCulloh, MD, Jacob K. Olson, MD, Yijie Wang, MS, Jennifer Vu, Sarah Gartner, BS, and Gail E. Besner, MD*

Department of Pediatric Surgery, Nationwide Children’s Hospital, Columbus, Ohio

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Abstract

Background: Necrotizing enterocolitis (NEC) is a leading cause of morbidity and mortality in premature infants. Increased intestinal permeability is central to NEC development. We have shown that stem cells (SCs) can reduce the incidence and severity of NEC. Our current goal was to investigate the efficacy of four different types of SC in preservation of gut barrier function during NEC.

Materials and methods: We compared (1) amniotic fluid-derived mesenchymal SC, (2) bone marrow-derived mesenchymal SC, (3) amniotic fluid-derived neural SC, and (4) enteric neural SC. Premature rat pups received an intraperitoneal injection of $2 \times 10^6$ SC or phosphate-buffered saline only and were then subjected to experimental NEC. Control pups were breastfed and not subjected to NEC. After 48 h, animals received a single enteral dose of fluorescein isothiocyanate-labeled dextran (FD70), were sacrificed 4 h later, and serum FD70 concentrations determined.

Results: Compared to breastfed, unstressed pups with intact gut barrier function and normal intestinal permeability (serum FD70 concentration $2.22 \pm 0.271 \text{ mg/mL}$), untreated pups exposed to NEC had impaired barrier function with significantly increased permeability ($18.6 \pm 4.25 \text{ mg/mL}$, $P = 0.047$). Pups exposed to NEC but treated with SC had significantly reduced intestinal permeability: Amniotic fluid-derived mesenchymal SC ($9.45 \pm 1.36 \text{ mg/mL}$, $P = 0.017$), bone marrow-derived mesenchymal SC ($6.73 \pm 2.74 \text{ mg/mL}$, $P = 0.049$), amniotic fluid-derived neural SC ($8.05 \pm 1.31 \text{ mg/mL}$, $P = 0.0496$), and enteric neural SC ($6.60 \pm 1.46 \text{ mg/mL}$, $P = 0.033$).

Conclusions: SCs improve gut barrier function in experimental NEC. Although all four types of SC reduce permeability equivalently, SC derived from amniotic fluid may be preferable due to availability at delivery and ease of culture, potentially enhancing clinical translation.

Introduction

Despite several decades of research, necrotizing enterocolitis (NEC) continues to be a leading cause of morbidity and mortality among premature infants. Several studies in our laboratory and in those of others have demonstrated a reduction in the incidence and severity of experimental NEC in vivo through treatment with amniotic fluid-derived...
mesenchymal stem cells (AF-MSC), bone marrow–derived MSC (BM-MSC), or enteric neural stem cells (E-NSC; Table). It has also been shown that intraperitoneal (IP) injection of conditioned medium from AF-MSC is effective in treating experimental NEC. We have recently compared the following four different types of stem cells (SCs) and shown them to all have equivalent efficacy in decreasing histologic injury during experimental NEC: AF-MSC, amniotic fluid–derived NSC (AF-NSC), BM-MSC, and E-NSC. However, no study has investigated the ability of different types of SCs to maintain gut barrier function. Our goal in this study was to determine whether different SC types vary in their ability to preserve intestinal mucosal integrity during experimental NEC.

**Materials and methods**

Four different types of SCs were compared: (1) AF-MSC, (2) BM-MSC, (3) AF-NSC, and (4) neonatal E-NSC (Fig. 1). All cell lines were derived from Lewis rats.

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<th>Table – Review of published studies that have shown beneficial effects of stem cell therapy in necrotizing enterocolitis.</th>
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EYFP = enhanced yellow fluorescent protein; EGFP = enhanced green fluorescence protein; HB-EGF = heparin-binding EGF-like growth factor; IV = intravenous; SD = Sprague–Dawley.
AF-MSC culture

Amniotic fluid (AF) was harvested from timed pregnant rats on Day 14.5 of gestation via aspiration of amniotic sacs using a 25 ga needle, based on previously described procedures.\textsuperscript{12,13} AF was placed into T175 flasks in culture media composed of Minimum Essential Medium Alpha with GlutaMAX (MEM-α; ThermoFisher, Waltham, MA), supplemented with 10% Embryonic Stem Cell Qualified Fetal Bovine Serum (ThermoFisher), 18% Chang B and 2% Chang C (Irvine Scientific, Santa Ana, CA), and 1% penicillin/streptomycin/amphotericin B (PSA; ThermoFisher). Cells were passaged in a 1:4 ratio upon reaching 80%-90% confluence by removing nonadherent cells and debris with phosphate-buffered saline (PBS), followed by addition of 0.25% Trypsin-ethylenediaminetetraacetic acid (Trypsin; ThermoFisher), which was inactivated after 90 s by addition of complete medium, followed by centrifugation at 400g for 5 min. Cells from passages 4-9 were used for experimental studies.

BM-MSC culture

Bone marrow was harvested from the femurs and tibias of dams at the same time as AF was harvested and cultured as previously documented.\textsuperscript{14-16} Bones were cleared of surrounding tissue and the ends clipped with bone cutting forceps. Marrow was flushed with MEM-α and placed directly into T175 flasks in culture media composed of MEM-α, 10% Mesenchymal Stem Cell Qualified Fetal Bovine Serum (ThermoFisher), and 1% PSA. Cells were passaged in a 1:4 ratio at 80%-90% confluence using the same process used for AF-MSC, with cells from passages 4-9 used in all experimental studies.

AF-NSC culture

AF was harvested as described for AF-MSC. Using a modification of previously described procedures, AF was placed into culture flasks containing NSC medium composed of Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (ThermoFisher), supplemented with 4% chicken embryo extract (Gemini Bio-Products, West Sacramento, CA), 2% PSA, 1X N-2 supplement (ThermoFisher), 20 ng/mL recombinant fibroblast growth factor basic (R&D Systems, Minneapolis, MN), and 20 ng/mL recombinant rat epidermal growth factor (R&D Systems).\textsuperscript{17,18} Medium was changed every 4 d, during which time NSC aggregates known as neurospheres formed. After 2 wk of growth, these neurospheres were then dissociated to individual NSC using a combination of chemical dissociation with StemPro Accutase (ThermoFisher) and mechanical dissociation via gentle pipetting.

Fig. 1 – Phase contrast microscopy of SCs, ×40. (A) AF-MSC; (B) BM-MSC; (C) AF-NSC; (D) E-NSC.
E-NSC culture

E-NSC were obtained from the small intestines of rat pups aged 4-7 d as in previous studies. Briefly, the entire small intestine was harvested from duodenum through ileum and opened longitudinally, allowing the mucosa and submucosa to be stripped away. The muscular layer was then digested using 1 mg/mL Collagenase and 1 mg/mL Dispase (Worthington Biochemical Corporation, Lakewood, NJ) in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 for 45-60 min. The resulting solution was filtered through a 70-μm filter, centrifuged, and resuspended in NSC medium. Media were changed every 4 d and observed for the formation of neurospheres that were processed in the same fashion as for AF-NSC.

SC verification

Flow cytometry was used to confirm known SC markers on all cell populations. AF-MSC were confirmed to be positive for CD29, CD49, CD90, and Oct4. CD29, CD49, and CD90 are surface markers of MSC derived from AF. Oct4 is a transcription factor suggestive of early stemness, and positivity of some cells was observed, as expected. These cells were also confirmed to be negative for CD11 and CD45 (surface markers of hematopoietic SCs). BM-MSC cells were confirmed to be positive for CD90, again indicating MSCs. These cells were also negative for CD11 and CD45. Both AF-NSC and E-NSC cell populations were confirmed via flow cytometry to be positive for the presence of Nestin, an intermediate filament protein transiently expressed only in NSC but not in mature cells of neural origin.

In addition, multipotency of AF-MSC and BM-MSC populations was confirmed via adipogenic and osteogenic induction using the StemPro Adipogenesis Differentiation Kit (ThermoFisher) and StemPro Osteogenesis Differentiation Kit (ThermoFisher) as previously described. Cells induced to form adipocytes stained positively using Oil Red O, confirming the presence of lipid droplets, and cells induced to form osteocytes stained positively using Alizarin Red S, indicating calcium deposition in cells induced to form osteocytes (Fig. 2).

Experimental NEC model

All animal studies were conducted in compliance with protocol #AR15-00012 approved by the Institutional Animal Care and Use Committee of The Research Institute at Nationwide Children’s Hospital. The model used in our laboratory has been extensively documented and is based on a modification of the original model of Barlow et al. Briefly, Sprague-Dawley rat pups were delivered via C-section approximately one-half day prematurely from timed-pregnant dams (Envigo, Indianapolis, IN). Pups were then randomized to one of six groups: (1) Breastfed (n = 10); (2) NEC + 1% PBS (Corning, Manasses, VA; n = 35); (3) NEC + AF-MSC (n = 57); (4) NEC + BM-MSC (n = 21); (5) NEC + AF-NSC (n = 24); (6) NEC + E-NSC (n = 22). All pups exposed to NEC received either a single IP injection of 2 x 10^6 SCs in PBS, or PBS alone. Pups were subjected to repeated episodes of hypoxic and hypothermic stress every 8 h. Hypoxia was induced by placement into a chamber containing N2 gas calibrated to FIO2 < 1.5% for a duration of 90 s. Pups were placed into a 4°C environment for 10 min to induce hypothermic stress. Additional stress was induced via hypercaloric gastric gavage feeds of Esbilac milk replacer (PetAg, Hampshire, IL) fortified with Similac 60/40 (Ross Pediatrics, Columbus, OH) every 4 h, for a total of 836.8 kJ/kg per day. On the first day of the model, pups also received a single oral dose of 2 mg/kg lipopolysaccharide (Sigma–Aldrich, St. Louis, MO). Breastfed pups were placed with a surrogate dam immediately after delivery and not exposed to experimental stresses.

Permeability assay

After 48 h, pups in all groups received a single enteral dose of fluorescein isothiocyanate-labeled dextran (FD70, molecular weight 70,000, 750 mg/mL; Sigma–Aldrich) in 100 μL of PBS.
Four hours later, all pups were sacrificed via decapitation and serum collected into BD Microtainer SST tubes (Becton, Dickinson and Company, Franklin Lakes, NJ), inverted a minimum of 20 times, and allowed to stand for a minimum of 30 min before centrifugation at 2000g for 15 min. The resulting cell-free serum was then diluted into 270 μL of PBS, mixed, and placed into three separate wells in a 96-well plate for reading in a fluorescent plate reader with filter cutoffs of 492/518 nm. Serum samples with a volume <50 μL were discarded and not analyzed. The values read in each of the three samples were averaged and compared with a standardized curve determined by serial dilutions of the original known-concentration FD70 solution to determine serum concentrations.

**Statistical analyses**

All values are reported as mean ± standard error of the mean. Statistical significance of FD70 levels was assessed using one-way analysis of variance followed by Student’s t-test for pairwise comparison. P values < 0.05 were considered statistically significant.

**Fig. 3** — Experimental design. Pups are delivered via C-section at time zero and exposed to a series of repeated stresses, including hypoxia, hypothermia, LPS, and hypercaloric gavage feeds. At the time of the final feed, pups are given a single dose of FD70 and 4 h later, animals are sacrificed and serum FD70 levels determined.

**Fig. 4** — Intestinal permeability during experimental NEC. Each bar represents the mean serum FD70 concentration in the different treatment groups, with error bars showing SEM. No significant differences were found when comparing each of the four SC treatment groups to one another.
Results

Breastfed pups not exposed to experimental NEC had intact gut barrier function with normal intestinal permeability (serum FD70 concentration: 2.22 ± 0.271 μg/mL; Fig. 4). Pups exposed to experimental NEC that received IP PBS alone had impaired gut barrier function with significantly increased intestinal permeability (18.6 ± 4.5 μg/mL, P = 0.0005). Compared with pups treated with PBS alone, pups treated with SC had significantly reduced intestinal permeability as follows: AF-MSC (9.45 ± 1.35 μg/mL, P = 0.017); BM-MSC (6.73 ± 2.74 μg/mL, P = 0.049); AF-NSC (8.052 ± 1.31 μg/mL, P = 0.0496); and E-NSC (6.60 ± 1.46 μg/mL, P = 0.033). When comparing each of the SC groups to one another, no significant differences were found in intestinal permeability.

Discussion

The morbidity and mortality of NEC continue to be significantly high despite improvements in neonatal care and improved understanding of the pathology of the disease. We have previously shown that the four SC types investigated in this study equivalently reduce the incidence and severity of NEC from 61% to 19%-23%. However, it remains unclear how these SCs that are from different sources lead to similarly improved outcomes. We have previously demonstrated that different types of SCs can affect different areas of the intestine, with transplanted NSCs localizing within the myenteric plexus rather than within regions of epithelial origin. Based on these observations, we sought to determine whether differences in SCs would lead to differences in mucosal barrier protection. However, the current study suggests that different types of SCs equivalently preserve intestinal barrier function through mechanisms that remain unclear. Zani et al. showed that AF-MSC exert their effects through a COX-2-dependent pathway that modulates the stromal cells themselves. We and others have previously demonstrated that engraftment levels of different types of SC (AF-MSC, BM-MSC, and E-NSC) are higher in injured tissues than noninjured tissues; however, overall engraftment rates are relatively low and may not account for the protective effects of SC even in injured tissues. We are currently investigating the patterns of engraftment between these different SC populations, with the goal of identifying differences in how these cells incorporate into intestinal tissue.

Our current results demonstrate that treatment with different types of SC preserves and maintains the integrity of the intestinal barrier during injury. However, it is unclear whether the enhanced gut barrier function observed is due to pathways that are shared or different between the different SC types. Previous studies have shown that SCs can modulate inflammatory cytokines during injury, which may result in improved healing and decreased apoptosis in injured tissues. These anti-inflammatory pathways may be essential to the improved intestinal barrier function seen in all cell types in this study.

Although we have shown that SC therapy can improve gut barrier function in NEC, there are a number of challenges posed with bringing this therapy to the bedside. SCs have the potential to trigger tumorigenic and immunologic responses in humans that present a significant challenge to translation. In addition, in previous studies, we have demonstrated significant entrapment of SCs in the lungs of newborn rat pups with intravenous administration. Because of many challenges faced by SC therapy, we are currently investigating the potential for non–cell-based therapies for NEC. One very attractive possibility is the use of SC-derived exosomes. Exosomes are nanovesicles ~40-100 nm in size that contain DNA, RNA, microRNA, and proteins. These nanovesicles are released by all cells in the body via exocytosis and act as intercellular messengers that can affect changes in both neighboring and remote cells throughout the body. We have previously shown that BM-MSC–derived exosomes have the same protective effect on the intestines during experimental NEC compared with BM-MSC themselves. Our future studies will investigate exosomes derived from different SC types to identify an optimal cell-free treatment for NEC.
Conclusions

All four SC types investigated in this study equivalently improve gut barrier function and reduce intestinal permeability in an animal model of NEC. This is consistent with prior experiments in which we have shown that all four of these types of SCs equivalently reduce the incidence and severity of experimental NEC. Although the efficacy of protection afforded by these different cells appears equivalent, further work is needed to elucidate the pathways and mechanisms by which these different SC types exert their effects to determine whether they are common or divergent between different types of SC. It is our hope that in the future, SC-based therapy using SCs themselves or their secreted products may be used to prevent damage to the intestine with loss of intestinal mucosal barrier function due to NEC (Fig. 5).

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Authors’ contributions: C.M. was responsible for design and implementation of the experiments and creation and revision of the article. J.O. contributed to implementation of the experiments and creation and revision of the article. Y.W. assisted with stem cell harvesting and review and revision of the article. J.V. and S.G. assisted with implementation of the NEC model and review and revision of the article. As principal investigator, G.B. oversaw the design and implementation of the experiment, and review and revision of the article.

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Disclosure

None of the authors have any conflicts of interest to disclose.

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