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Stem Cells and Necrotizing Enterocolitis: A Direct Comparison of the Efficacy of Multiple Types of Stem Cells

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Abstract

Purpose—Necrotizing enterocolitis (NEC) is a leading cause of gastrointestinal morbidity and mortality in premature infants. While studies have shown potential for stem cell (SC) therapy in experimental NEC, no study has compared different SC side-by-side. Our purpose was to determine whether one type of SC may more effectively treat NEC than others.

Methods—Four SC were compared: (1) amniotic fluid-derived mesenchymal SC (AF-MSC); (2) amniotic fluid-derived neural SC (AF-NSC); (3) bone marrow-derived mesenchymal SC (BM-MSC); and (4) neonatal enteric neural SC (E-NSC). Using an established rat model of NEC, pups delivered prematurely received an intraperitoneal injection of SC. Control pups were injected with PBS. Additional controls were breast-fed by surrogates and not subjected to experimental NEC. Intestinal tissue was graded histologically.

Results—NEC incidence was: PBS, 61.3%; breast-fed unstressed, 0%; AF-MSC, 19.1%; BM-MSC, 22.9%; AF-NSC, 18.9%; E-NSC 22.2%. All groups demonstrated statistical significance ($p < 0.05$) compared to controls, and there was no difference between SC groups.

Conclusion—All four SC groups reduced the incidence and severity of experimental NEC equivalently. AF-MSC may be preferable due to availability of AF at delivery and ease of expansion, increasing potential for clinical translation.

Keywords

NEC; necrotizing enterocolitis; stem cells; amniotic fluid; mesenchymal; neural stem cells

Necrotizing enterocolitis (NEC) is a leading cause of gastrointestinal morbidity and mortality in premature infants.(1) Stem cell therapy has been shown in *in vivo* animal models to protect the intestines from several different types of injury including NEC.(2–9) While previous studies have shown promise with administration of stem cells, no study has directly compared the efficacy of different types of stem cells side-by-side. Our purpose was

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to determine whether stem cells from four different sources have varying efficacies, enabling the identification of stem cell(s) that may more effectively treat NEC.

1. Methods

1.1 Cell Culture

All cell cultures were derived from timed-pregnant Lewis rats (Rat Resource and Research Center, University of Missouri, Columbia, MO), sacrificed at E14.5 of gestation (estimated day 14.5 of an average 22 day gestation).

AF-MSC cells were obtained using modifications of previously described procedures.(10, 11) AF was harvested via 25ga needle aspiration of amniotic sacs. Cells were cultured in Minimum Essential Medium Alpha with GlutaMAX™ (MEM-α, ThermoFisher, Waltham, MA), supplemented with 10% Embryonic Stem Cell Qualified Fetal Bovine Serum (ES-FBS, ThermoFisher), 18% Chang B and 2% Chang C (Irvine Scientific, Santa Ana, CA), and 1% penicillin/streptomycin/amphotericin B (PSA, ThermoFisher). Media was changed every three days. Cells were routinely passaged using 0.25% Trypsin-EDTA (Trypsin, ThermoFisher) at 80–90% of confluence. All cells used for studies were from passages 4–9.

AF-NSC primary cell cultures were established from AF, with NSC induction performed using modifications of previously described protocols.(12, 13) Cells were cultured in NSC growth medium consisting of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, ThermoFisher) supplemented with 4% chicken embryo extract (Gemini Bio-Products, West Sacramento, CA), 2% PSA, 1X N-2 supplement (ThermoFisher), 20 ng/ml recombinant rat fibroblast growth factor basic (FGF, R&D Systems, Minneapolis, MN), and 20 ng/ml recombinant rat epidermal growth factor (EGF, R&D Systems). NSCs grew in this medium as cellular aggregates known as neurospheres. Media was changed every four days.

BM-MSC primary cell cultures were established from marrow harvested from femurs and tibias as previously described.(14–16) Bones were dissected of surrounding tissue and marrow flushed with culture media through a 25ga needle. Culture media was composed of MEM-a with 10% MSC-Qualified Fetal Bovine Serum (MSC-FBS, ThermoFisher), and 1% PSA. The first media change occurred after five days, and subsequently every three days. Cells were passaged using Trypsin at 80–90% confluence. All cells used for studies were from passages 4–9.

E-NSC primary cell cultures were established from neonatal rat pups 4–7 days after birth using a previously described method.(6, 17–19) Intestine from duodenum to ileum was harvested, opened longitudinally, and the mucosa and submucosa stripped away. The muscular layer was digested in DMEM/F12 containing 1mg/mL Collagenase and 1mg/mL Dispase (Worthington Biochemical Corporation, Lakewood, NJ) for 45 – 60 min. Cells were passed through a 70µm filter, centrifuged, resuspended in NSC growth medium, and observed for the formation of neurospheres. Media was changed every four days.

1.2 Stem Cell Identification

Flow cytometry was used to investigate known SC markers (Figure 1). AF-MSCs (passage 6) were investigated for positivity of CD29 (ThermoFisher), CD49e (ThermoFisher), CD90 (ThermoFisher), and Oct4 (Novus Biologicals, Littleton, CO), and negativity of CD11 (ThermoFisher) and CD45 (ThermoFisher). BM-MSCs (passage 6) were investigated for positivity of CD90, and negativity of CD11 and CD45. AF-NSC and E-NSC were examined for Nestin (R&D Systems) positivity.

AF-MSC and BM-MSC were examined for multipotency (Figure 2). StemPro Adipogenesis and Osteogenesis Differentiation Kits (ThermoFisher) were utilized to induce passage 5 cells to form adipocytes and osteocytes. Adipocytes were stained with Oil Red O. Osteoblasts were verified with Alizarin Red S.

1.3 Experimental NEC Model

All research followed ethical guidelines under IACUC-approved protocol #AR15-00012 at The Research Institute at Nationwide Children's Hospital. The model of NEC was based on that described by Barlow *et al.* (6, 7, 20–23) Rat pups delivered via C-section one half-day prematurely were randomized into six groups: (1) breastfed ($n = 10$); (2) NEC + 1% phosphate-buffered saline (PBS, Corning, Manassas, VA) ($n = 62$); (3) NEC + AF-MSC ($n = 42$); (4) NEC + AF-NSC ($n = 37$); (5) NEC + BM-MSC ($n = 48$); (6) NEC + E-NSC ($n = 36$). After delivery, each pup received a single intraperitoneal (IP) injection of 2×10^6 SC in 0.1 mL PBS. Pups in the NEC + PBS group received a single IP injection of 0.1 mL PBS. Every 4h, pups received orogastric feeds of Esbilac milk replacer (PetAg, Hampshire, IL) fortified with Similac 60/40 powder (Ross Pediatrics, Columbus, OH) to provide 836.8 kJ/kg per day. Feeds were initiated at 0.1 mL per feeding on day 1, and increased by 0.1 mL per day to 0.4 mL per feeding on day 4. At the time of the second feeding, pups also received orogastric delivery of 2 mg/kg lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO). (22, 24) Pups were exposed to hypoxic and hypothermic stress every 8 hours. Hypoxia was induced via exposure to 100% N₂ gas, ensuring FiO₂ < 1.5% for 90 seconds. Hypothermia was induced by placement into a 4°C environment for 10 minutes. Breastfed control pups were placed with surrogate dams immediately following C-section and not subjected to experimental stress. Pups were sacrificed at 96 hours post-delivery or upon development of signs of NEC (bloody stools, abdominal rigidity or distention, or respiratory distress).

1.4 Histologic Grading

At the time of sacrifice, three pieces each of duodenum, jejunum, and ileum were fixed in 10% formalin (Fisher Scientific, Pittsburgh, PA) for 24h, paraffin-embedded, cut into 5 µm sections, and stained with hematoxylin and eosin (H&E). Two independent blinded reviewers assessed damage using the grading scale described by Caplan *et al.* as follows: 0, normal tissue; 1, epithelial cell lifting; 2, necrosis to mid-villus; 3, necrosis of entire villus; 4, transmural necrosis (Figure 3). Injury grades 2, 3 and 4 were considered consistent with NEC. (25)

1.5 Statistical Analyses

Statistical significance of NEC incidence and severity were determined via Chi-square or Fisher's exact test, where appropriate. Significance of NEC incidence and severity between breastfed and NEC + PBS was determined using Welch's t-test. P values < 0.05 were considered significant.

2. Results

2.1 NEC Incidence and Severity

No breastfed control pups developed NEC (0%) (Figure 4). Compared to breastfed pups, 61.3% of pups exposed to experimental stress that received PBS alone developed NEC ($p < 0.0001$). Compared to pups that received PBS, pups that received SC had the following incidences of NEC: AF-MSCs 19.1% ($p < 0.0001$); BM-MSCs 22.9% ($p < 0.0001$); AF-NSC 18.9% ($p < 0.0001$); E-NSC 22.2% ($p = 0.0002$). No significant difference was found between different SC types with respect to ability to decrease experimental NEC.

3. Discussion

Although recent advances have significantly improved survival of premature infants at younger gestational ages and weights, the morbidity and mortality of NEC remain unacceptably high.(26) Numerous *in vivo* animal studies have demonstrated success with individual types of stem cells. BM-MSCs and E-NSCs have been shown in multiple studies, including our own, to be effective at reducing experimental NEC.(5–7, 23) Zani *et al.* have shown that AF-MSCs are effective in treating experimental NEC and in reducing associated ascites via COX-2-dependent modulation of stromal cells.(3, 8) While SC were initially thought to home to and engraft into injured tissues, this is unlikely to be the case. Although engraftment occurs to some degree in various models, it is often short-lived, and the beneficial effects are more likely due to signaling mechanisms that enhance repair and modulate inflammation.(8, 27, 28)

Our current study demonstrates that four different types of SC significantly reduce the incidence and severity of experimental NEC, and there is no significant difference in the ability of the different SC types to exert their effects. Still unclear is how these different SC exert their effects. An exciting area of investigation is exosomes and their role in cellular communication. We have previously shown that exosomes derived from BM-MSCs are as effective in treating NEC as the BM-MSCs themselves.(23) It has also been shown that IP injection of conditioned medium from AF-MSCs, which contains a mix of cellular messengers including exosomes, has the same effect as injection of the SC themselves.(8)

There are some limitations to the current study. Previous animal studies of stem cell therapy for NEC examined individual cell types from varying donor species using various cell numbers. Since previous studies from our laboratory and others using a similar rat model of NEC demonstrated that 2×10^6 AF-MSCs were effective in reducing the incidence and severity of NEC, and that IP injection of this number of cells is safe in rats, we chose to use this quantity of cells for comparison of different stem cell types.(8, 29) Future research will include studies to determine whether a dose-response phenomenon exists that may allow for

further optimization of stem cell therapy. Additionally, the current study does not provide insight into the mechanisms by which these stem cells exert their effects, and studies addressing this are currently underway.

SC therapy must overcome several challenges before translation to the bedside. Although no adverse effects were observed with IP injection of stem cells in the current study, we have previously shown that intravenous injection of BM-MSC leads to significant entrapment of SC in the lungs.(30–32) Additional significant risks of SC therapy include immunogenic and tumorigenic response.(33) Cell-free treatment with exosomes has the potential to target specific tissues without the risk of tumor formation associated with SC.(34) We plan to characterize exosomes derived from these different SC types and investigate their efficacies. While common inflammatory mediators may underlie their effects, it is possible that their mechanisms of action are varied. Our current and planned studies should provide further insight that allows optimization of SC therapy for NEC.

4. Conclusions

In this study, different types of stem cells (AF-MSC, BM-MSC, AF-NSC, or E-NSC) significantly reduce the incidence and severity of NEC to an equivalent degree. Future use of AF-MSC may be preferable due to the availability of amniotic fluid at delivery and the ease with which AF-derived cells can be cultured. This consideration may be important for future clinical translation.

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Figure 1. Flow cytometric analysis of stem cells

Red lines represent negative controls and blue lines represent populations of interest. (A) AF-MSC, positive for CD29, CD49e, CD90; partially positive for Oct4; negative for CD11 and CD45; (B) BM-MSC, positive for CD90 and negative for CD11 and CD45; (C) AF-NSC, positive for Nestin; (D) E-NSC, positive for Nestin.

Figure 2. Multipotency verification

AF-MSC and BM-MSC (passage 5) were induced along adipogenic and osteogenic differentiation pathways. Shown are representative images of (A) AF-MSC with lipid droplets staining positive with Oil Red O after 7d and (B) AF-MSC staining positive for calcium with Alizarin Red S after 14d. Similar findings were obtained for BM-MSC.

Figure 3. Representative images of histologic injury scores

Sections of small intestine were stained with H&E and graded as follows: (A) grade 0, normal intestine; (B) grade 1, epithelial cell lifting; (C) grade 2, necrosis to mid-villus; (D) grade 3, necrosis of entire villus; (E) grade 4, transmural necrosis. Magnification $\times 20$.

Figure 4. Incidence and severity of NEC

With the exception of rat pups in the breastfed group, all other pups were subjected to experimental NEC. (A) Percentages of pups with histologic injury scores of 2, 3 or 4, consistent with histologic NEC. The white numbers within the colored sections of the bars represent the percent incidence of Grade 2, 3 or 4 NEC, and the bold numbers at the top of each bar represent the total incidence of NEC.

(B) Percentages of pups with histologic injury scores of 3 or 4, consistent with severe NEC. The white numbers within the colored sections of the bars represent the percent incidence of Grade 3 or 4 NEC, and the bold numbers at the top of each bar represent the total incidence of severe NEC.