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Partial or complete coverage of experimental spina bifida by simple intra-amniotic injection of concentrated amniotic mesenchymal stem cells

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ABSTRACT

Purpose: We sought to determine whether simple intra-amniotic delivery of concentrated amniotic mesenchymal stem cells (afMSCs) may elicit prenatal coverage of experimental spina bifida.
Methods: Time-dated pregnant Sprague–Dawley dams (n=24) exposed to retinoic acid for the induction of fetal neural tube defects were divided in three groups. Group I had no further manipulations. Groups II and III received volume-matched intra-amniotic injections of either saline (Group II) or a suspension of syngeneic afMSCs labeled with green fluorescent protein (Group III) in all fetuses (n=202) on gestational day 17 (term=21–22 days). Animals were killed before term. Statistical comparisons were by ANOVA ($P<0.05$).
Results: Of 165 fetuses viable at euthanasia, a spina bifida was present in 58% (96/165), with no significant differences in defect dimension across the groups ($P=0.19$). However, variable degrees of coverage of the defect by a rudimentary skin confirmed histologically were only present in Group III ($P<0.001$), in which donor afMSCs were documented, with no differences between Groups I and II ($P=0.98$).
Conclusions: Amniotic mesenchymal stem cells can induce partial or complete coverage of experimental spina bifida after concentrated intra-amniotic injection. Trans-amniotic stem cell therapy (TRASCET) may become a practical option in the prenatal management of spina bifida.

Congenital neural tube defects (NTDs) stem from the failure of the neural tube to close by the fourth week of embryonic development. These defects may involve any portion of the brain and/or spinal cord. Spina bifida is the most common NTD compatible with life. Despite mandatory folate supplementation in all enriched cereal grain products, the incidence of spina bifida in the United States has remained relatively stable over the last several years, at 3–4 per 10,000 live births [1].

The spinal cord damage associated with spina bifida is thought to be both primary, resulting from abnormal spinal cord development, as well as secondary, resulting from spinal cord exposure to the amniotic fluid (chemical insult) and local trauma (mechanical insult). Several studies suggest that the secondary component is the most relevant, justifying prenatal surgical coverage of the defect in select cases. While fetal surgery has proven valuable in a subset of fetuses with this defect, it is not indicated in the majority of them and it is not without significant maternal and fetal risks, most notably preterm labor.

In this study, we sought to test the hypothesis that coverage of the spina bifida defect may be induced non-surgically, specifically via therapeutic manipulation of a recently described component of the unique fetal wound healing process, namely the amniotic fluid-derived mesenchymal stem cell (afMSC).

1. Methods

This study was approved by the Boston Children's Hospital Institutional Animal Care and Use Committee under protocol #12-05-2186R.

1.1. Congenital NTD model

Twenty-four time-dated pregnant Sprague–Dawley dams (Charles River Laboratories, Inc., Wilmington, MA) were fed a normal diet *ad libitum* and housed under a standard dark/light cycle. All animals were submitted to the induction of neural tube defects in their fetuses as previously described [2]. Briefly, after exposure to isoflurane (Abbot Laboratories, North Chicago, IL), chamber inhaled at 2%–4% in 100% oxygen, the dams received 60 mg/kg of all-trans retinoic acid (Sigma-Aldrich Chemical, St. Louis, MO), dissolved in olive oil at 10 mg/mL at room temperature, through gavage, on gestational day ten (E10; term=21–22 days), purposely between 6:00 p.m. and 8:00 p.m. Animals were then divided in three groups. Group I had no further manipulations. Groups II and III received volume-matched intra-amniotic injections of either saline (group II), or a suspension of afMSCs (group III) blindly in all viable fetuses later in gestation, as described in detail below.

1.2. Amniotic fluid procurement and donor afMSC processing

Donor afMSCs consisted of previously banked cells from normal syngeneic Lewis rat dams that served purely as amniotic fluid donors on E21. A midline laparotomy was performed and the bicornuate uterus

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eviscerated. Amniotic fluid from viable fetuses was obtained using a 30G needle (Becton Dickinson, Franklin Lakes, NJ) on a 1 mL syringe (Becton Dickinson) introduced into each amniotic cavity upon the ventral aspect of the fetus, through which amniotic fluid was procured.

A combination of mechanical separation and natural selection with culture media was used to isolate afMSCs from the amniotic samples, based on the methods as we have previously described [3–5]. Fluorescence-activated cell sorting analysis was used to confirm their mesenchymal progenitor identity with unconjugated mouse monoclonal antibodies previously validated for use in rats, namely for CD29 (Becton Dikinson, East Rutherford, NJ); CD44 (Becton Dikinson); CD45 (Becton Dikinson); CD73 (Becton Dikinson); and CD90 (Becton Dikinson), using the Vantage SE cell sorter (BD Biosciences). A mouse isotype immunoglobulin control was used to exclude nonspecific staining. Cells were labeled with green fluorescent protein (GFP) via retroviral infection using a pCL10A1 packaging vector and pMIG plasmid. GFP positivity was confirmed via FACS analysis at 89% at passage 4 and retained its integrity throughout the course of the experiment (77% at passage 16).

1.3. Intra-amniotic injections

The Sprague–Dawley dams from Groups II and III received their intra-amniotic injections on E17. General anesthesia was induced and maintained with isoflurane (Abbot), chamber inhaled at 2%–4% in 100% oxygen. A large midline incision was made and the bicornuate uterus was eviscerated. A 33G non-coring needle (Hamilton Company, Reno, NV) on a 100 μL syringe (Hamilton Company) was introduced into each and every amniotic cavity containing a viable fetus (n=202) by the ventral aspect of the fetus, carefully avoiding it and the umbilical cord (Fig. 1). Depending on the group, each injection consisted of either 50 μL of simple PBS (Group II), or 50 μL of a suspension of the labeled syngeneic afMSCs (Group III), at 2×10^6 cells/mL in PBS. The uterus was returned to the abdomen and the incision closed in two layers with 3-0 Prolene (Ethicon, Somerville, NJ) and 5-0 Vicryl (Ethicon) simple running sutures. Animals were allowed to recover with no additional manipulations other than post-operative analgesia with buprenorphine (Reckitt and Colman Pharmaceuticals, Richmond, VA) as needed.

1.4. Spina bifida defect analysis

Dams from all three groups were euthanized with chamber-inhaled carbon dioxide just prior to term, on E21. A midline incision was made and the uterus was eviscerated. Each amniotic cavity was incised and the fetus examined for the presence or absence of an NTD. Defects were tabulated as either isolated spina bifida, isolated exencephaly, or a combination of the two. Fetal weight was measured with an NBL 124e scale (ADAM Equipment, Danbury, CT). The overall fetal crown-rump length, as well as the longest cranial–caudal and lateral dimensions of the spina bifida defects, was measured using a digital vernier caliper (Thermo Fisher Scientific, Waltham, MA) calibrated as per manufacturer instructions. The presence or absence of any discernible degree of coverage of the spina bifida defect was scrutinized in all fetuses by both gross inspection and histology.

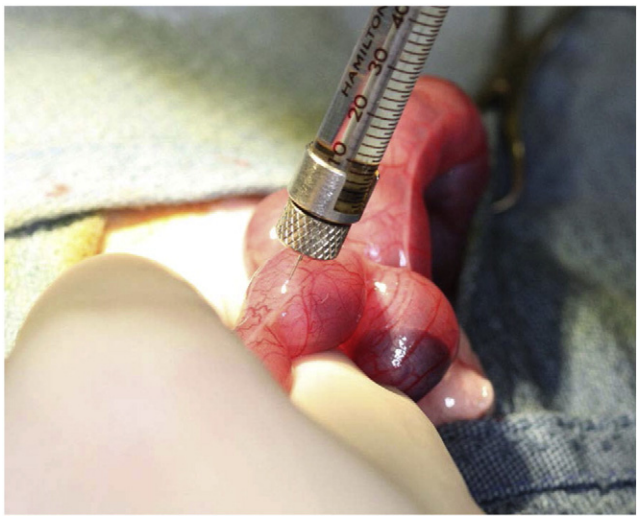

Fig. 1. Gross view of the intra-amniotic injection by the ventral aspect of the fetus, carefully avoiding it and the umbilical cord, using a 33G non-coring needle on a 100 μL syringe.

Histomorphologic evaluations were performed in paraffin-embedded specimens only at the level of the spina bifida defect. Sections were then stained with hematoxylin–eosin (H&E). Due to autofluorescence in control specimens and our goal of precise engraftment site screening, detection of labeled donor cells was by immunohistochemistry for GFP performed on fixed specimens using a murine monoclonal anti-GFP antibody (polyclonal, Abcam, Cambridge, MA) at 1:1000 dilution, including exclusion of nonspecific cell staining with a mouse isotype immunoglobulin control, as detailed elsewhere [6]. Secondary detection was performed with the Leica Bond Polymer Refine detection kit (Leica, Chicago, IL), per manufacturer's instructions. All histological examinations were performed under a light microscope (Carl Zeiss, Jena, Germany) specially equipped for use with a digital camera (Canon Powershot S3 IS, Canon USA, Lake Success, NY).

1.5. Statistical analysis

Presence or absence of NTD creation and of spina bifida coverage (full or partial) – defined as confirmed both grossly and histologically – were compared between the three groups using a generalized estimating equations (GEE) logistic regression model with a binomial distribution accounting for varying numbers of fetuses within the same mother and the Wald test to assess significant differences between groups. Exact binomial 95% confidence intervals (CI) were calculated for presence of NTDs to give precision of the observed incidence. Linear mixed model nested ANOVA was used to compare defect size between the three groups to account for varying numbers of fetuses within the same mother with the Wald test used to assess significance between groups and pairwise Bonferroni comparisons to control type I errors. Comparisons in defect size between groups were also made adjusting for overall crown-rump length. Statistical significance was set at two-tailed $P<0.05$.

2. Results

A total of 165 fetuses from all three groups were viable at euthanasia. An NTD was present in 58% (96/165) of them, distributed as follows: Group I: 20/25 = 80% (95% CI: 61%–91%); Group II: 36/53 = 68% (95% CI: 55%–79%); and Group III: 40/87 = 46% (95% CI: 35%–55%). This distribution represented a statistically significant increase in the incidence of NTDs in Group I when compared with Group III ($P=0.03$), with no significant differences between Groups I and II ($P=0.39$) or between Groups II and III ($P=0.12$).

Overall, among the fetuses with NTDs, the vast majority (94%; 90/96) had a spina bifida, either isolated (87%; 78/90) or in combination with an exencephaly (13%; 12/90), with no statistically significant differences in distribution across the groups ($P=0.65$). Comparably, there were no significant differences across the groups in the cranial–caudal and lateral dimensions of the spina bifida defect, both in absolute values and when adjusted for overall fetal crown-rump length (Table 1).

In fetuses with spina bifida, although animals from all groups often displayed a thin fibrous membrane covering the defect, as described in the original retinoic acid model [2], a conspicuous difference among the groups was in the rate of variable degrees of coverage of the defect (partial or complete) by a membrane which appeared to be quite different from that previously described, shown to be a rudimentary skin, with a paucity (or lack) of adnexa, on histology (Figs. 2 and 3).