



Introduction

Myelomeningocele (MMC), or spina bifida, results from incomplete closure of the spinal cord and spinal canal during gestation, leaving the spinal cord unprotected. Intrauterine chemical and mechanical trauma to the exposed spinal cord results in lifelong lower limb paralysis, bowel and bladder dysfunction, musculoskeletal deformities, and severe cognitive disabilities due to hindbrain herniation in afflicted children. It is the most common congenital cause of lifelong paralysis in the United States, and approximately four children a day are born with this devastating congenital defect.

Previously, our lab discovered that treatment with early gestation placenta-derived mesenchymal stem cells (PMSCs) seeded on a clinical grade extracellular matrix (ECM) during in utero repair of MMC functionally cures paralysis in a rigorous ovine model, resulting in 75% improvement over standard in utero repair [5].

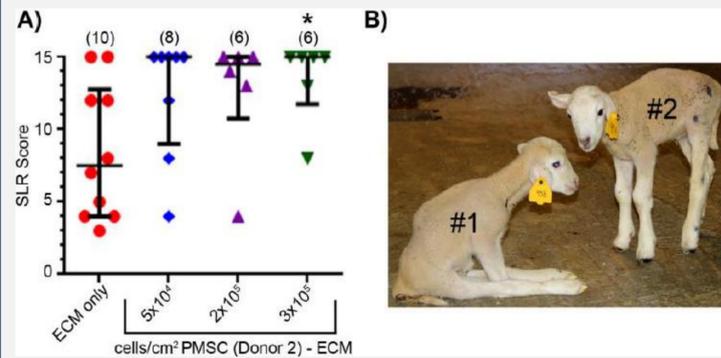


Figure 1. PMSCs seeded at different densities on ECM improved motor function at birth in the fetal ovine model of MMC

The neuro-restorative effects of mesenchymal stem cells are well documented and include promoting neurite outgrowth, secreting anti-apoptotic and neuroprotective factors, and regulating inflammation [6, 7]. In an in vitro neuronal protection assay, we found that PMSCs can rescue apoptotic neurons with their robust paracrine secretions [1]. Previously, we focused on neurotrophic growth factors including brain-derived neurotrophic factor (BDNF) and hepatocyte growth factor (HGF). However, less research has been conducted to determine the inflammatory regulation that PMSCs might incur in our ovine model. Studying the changes in the inflammatory response will help us to further ascertain the exact PMSC mechanism of action and provide a deeper understanding of our stem cell treatment.

Objectives

The purpose of this study was to determine if PMSC's regulate inflammation during neuroprotection in our fetal ovine model of myelomeningocele.

We hypothesize decreased inflammatory marker expression, specifically Glial Fibrillary Acidic Protein and vimentin (expressed in glial cell activation), in spinal cord treated with PMSC's compared to ECM only controls.

Methods

Immunofluorescence staining was performed 20 μm thick transverse sections of spinal cord at the epicenter of insult (most compressed). To block nonspecific binding, the slides were incubated with PBS containing 5% goat serum and 0.2% Tween 20 for 1 hour at room. The slide was washed with PBS and then incubated with primary antibody overnight at 4°C. The primary antibodies were used anti-Glial Fibrillary Acidic Protein (GFAP, 1:100, Abcam), anti-vimentin (1:100, Sigma), Immunofluorescence detection was performed with Alex Fluor 647 goat anti-mouse or Alex Fluor 555 goat anti-rabbit (1:1000; Invitrogen, Carlsbad, CA) and DAPI (ThermoFisher Scientific, Carlsbad, CA) for the nuclear staining. Adjacent tissue sections where the primary antibody was omitted served as negative controls.

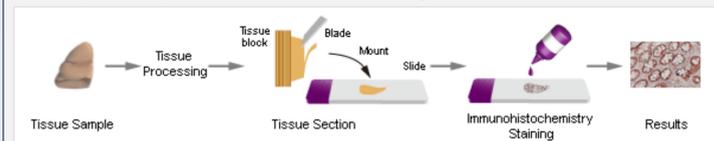


Figure 2. Immunohistochemistry schematic

Microscope images was acquired with an Axio Observer 200M inverted microscope and a monochromatic camera and arc lamp illumination using selective filters for fluorescence. Images was analyzed using Image J software. Fluorescence was quantified as the integrated fluorescence intensity per linear μm from 10 randomly acquired microscopy (200X) images of stained spinal cord cross sections. To account for variance of tissue autofluorescence, the ratio of the average fluorescence intensity of sections stained with primary antibodies to the negative controls were used for statistical analysis.

Results

Study Group	# Animals	Dose
Control	7	ECM Only
Treatment	9	3x10 ⁵ cell/cm ² ECM

Table 1. Study Design

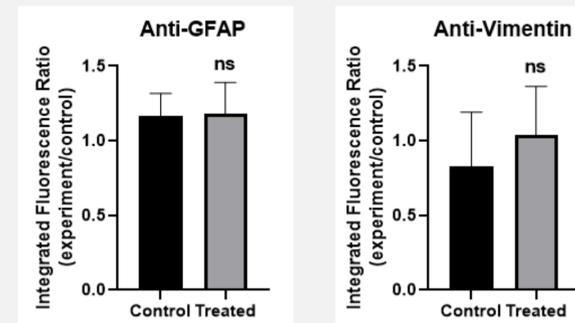


Figure 3. There was no significant differences in GFAP and vimentin expression demonstrated between treated and control groups.

Results Continued

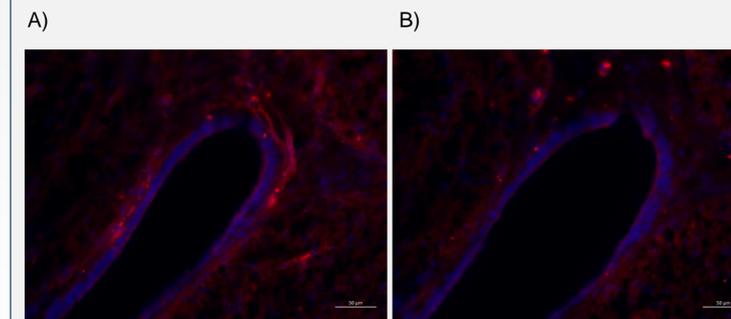


Figure 4. Representative GFAP Staining which highlights distinct GFAP expression around the periphery of a blood vessel (A). No distinct staining was discernable in the negative control (B).

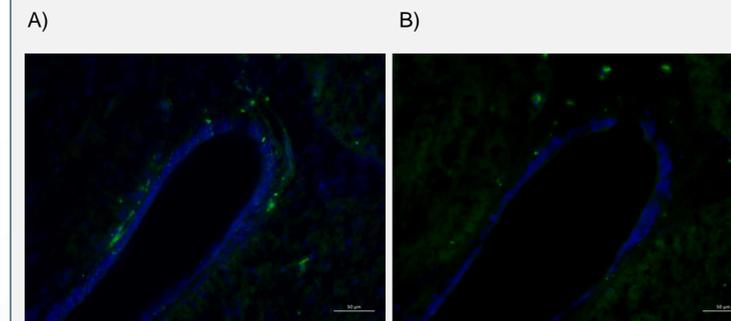


Figure 5. Representative Vimentin Stain which produced a similar pattern to the GFAP stain (A). No distinct pattern was discernable in the negative control (B).

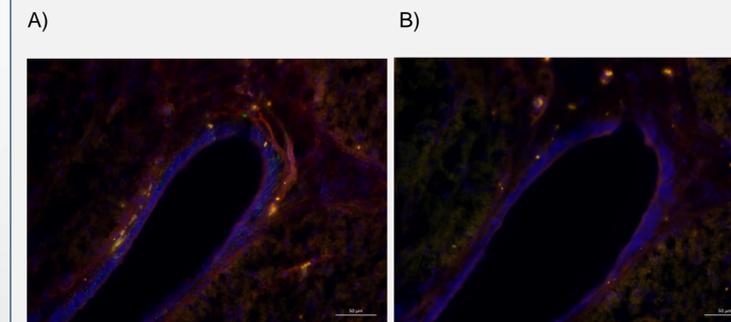


Figure 6. Representative Dual Staining which demonstrates colocalization represented by yellow which is the overlay of red GFAP and green vimentin signals (A). No distinct pattern was discernable in the negative control (B).

Conclusions

Our study indicate insignificant changes in inflammatory marker expression in spinal cord tissue treated with PMSCs. Immunofluorescence patterns suggest no difference in inflammatory response by glial cells, however, notable concerns exist:

- Fluorescence staining is susceptible to artifacts of variable fixation and tissue section thickness, as well as procedural details including consistent washing and sample handling.
- RBCs and clots contribute significant autofluorescence that can create false positive fluorescence and should be avoided during imaging.
- Euthanasia at 45 days following in-utero repair may or may not be optimal to observe inflammation in these studies. Timing of the inflammatory response needs to be further investigated.
- Antibodies for sheep tissue are not well characterized and are very difficult to titrate.
- Other pathways of immune modulation may exist that are not encompassed by this study

In conclusion, methodological approaches need to be further optimized, however preliminary data suggests no difference in the inflammatory response in spinal cord tissue treated with PMSCs in an ovine model of MMC.

References

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