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Surviving Lambs with Myelomeningocele Repaired in utero with Placental Mesenchymal Stromal Cells for 6 Months: A Pilot Study

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Abstract
Background: Fetal repair of myelomeningocele (MMC) with placental mesenchymal stromal cells (PMSCs) rescues ambulation in the ovine model up to 48 h postnatally. Outcomes past 48 h are unknown as MMC lambs have not been survived past this timepoint. ***Objective:*** We aimed to survive lambs for 6 months following the fetal repair of MMC with PMSCs. ***Methods:*** Fetal MMC lambs were repaired with PMSCs. Lambs received either no additional treatment or postnatal bracing and physical therapy (B/PT). Motor function was assessed with the sheep locomotor rating (SLR). Lambs with an SLR of 15 at birth were survived for 6 months or until a decline in SLR less than 15, whichever came first. All lambs underwent a perimortem MRI. ***Results:*** The lambs with no postnatal treatment ($n = 2$) had SLR declines to 7 and 13 at 29 and 65 days, respectively, and were euthanized. These lambs had a spinal angulation of 57° and 47°, respectively. The B/PT lamb ($n = 1$) survived for 6 months with a sustained SLR of 15 and a lumbar angulation of 42°. ***Conclusion:*** Postnatal physical therapy and bracing counteracted the inherent morbidity of the absent paraspinal muscles in the ovine MMC model allowing for survival and maintenance of rescued motor function of the prenatally treated lamb up to 6 months.

Introduction

The Management of Myelomeningocele Study established fetal repair of myelomeningocele (MMC) as the standard of care [1]. Despite significant improvement in motor function, only 55% of patients repaired prenatally were able to walk independently at the 30-month follow-up [2]. This inspired a search for a novel therapy to augment the fetal MMC repair to improve distal motor function and other clinical outcomes.

The ovine model is the most commonly used large animal model to study fetal MMC repair as sheep have a robust ability to tolerate multiple fetal interventions [3–7]. It is a surgical model that is morbid by design, with

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removal of all lumbar paraspinal muscles and lamina to create the defect and prevent innate healing in utero. The morbidity of absent paraspinal muscles and protective lamina makes survival of the lambs past 48 h of life challenging. We have previously shown that augmenting the standard fetal MMC repair in the ovine model with early gestation placental mesenchymal stromal cells (PMSCs) engineered in neurogenic media rescues ambulation in lambs up to 48 h of life [8]. This timepoint was previously selected as it was easily feasible across the many experiments and provided a consistent point of comparison. However, the durability of the improved motor outcomes past 48 h of life, as the lambs grow and gain weight similar to humans with MMC, remains unknown.

To determine the durability of the augmented repair, we aimed to survive lambs that underwent fetal MMC repair with PMSCs for 6 months. We hypothesized that the improved motor outcome of the augmented repair was durable for 6 months with improved motor outcome indicated by a motor function score of 15 on the sheep locomotor rating (SLR).

Methods

Placental Mesenchymal Stromal Cell Isolation and Seeding

PMSCs were explanted from donated placentas as described by Lankford et al. [9]. Cells were cultured in media consisting of Dulbecco's Modified Eagle's Medium/High Glucose, 5% fetal bovine serum (Hyclone, Thermo Fisher Scientific), 100 units/mL penicillin, 100 μg/mL streptomycin (Thermo Fisher Scientific), and 20 ng/mL recombinant human epidermal growth factor (R&D Systems).

The PMSCs were seeded at passage 5 onto a 6 × 2 cm piece of extracellular matrix (ECM) (Biodesign® Dural graft, Cook Biotech, West Lafayette, IN, USA) the day prior to MMC repair. Each fetus received a different PMSC line. A cell density of 500,000 cells/cm^2 was used for the first lamb and 300,000 cells/cm^2 for subsequent lambs. The PMSC-ECM product was incubated in culture media overnight the night prior to surgery. Two different imaging techniques were used. To prepare the ECM for the first 2 lambs, the PMSCs were transduced with green fluorescent protein-containing lentiviral vector (University of California, Davis Stem Cell Center, California Institute of Regenerative Medicine, Sacramento, CA, USA) at passage 4, then imaged the morning of the MMC repair to verify PMSC adherence to the ECM. To prepare the ECM for the third lamb, an additional ECM punch-out was seeded at the same time as the ECM and incubated with the PMSC-ECM product overnight, then stained with Calcein AM (Thermo Fisher Scientific) the morning of the MMC repair to verify PMSC adherence to the ECM.

Ovine Myelomeningocele Defect Creation, Repair, and Cesarean Delivery

Animal work was approved by the Institutional Animal Care and Use Committee, and care was in compliance with the Guide for the Care and Use of Laboratory Animals. The facilities used to conduct this study were accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

As previous described, the fetal MMC defect was created at 75 days of gestation ±7 days [10]. The skin, paraspinal muscles, vertebral lamina, and dorsal portion of the dura were resected from L1 to L6. A myelotomy was not performed because this study targeted evaluating motor function rather than hindbrain herniation. Lost amniotic fluid was replaced with normal saline, and antibiotics (100 mg of gentamicin and 1 million units of penicillin) were added to the amniotic fluid at the time of uterine closure.

As previously described, the MMC defect was repaired at 100 days of gestation ±7 days [10]. The fibrinous scar over the spinal cord was removed. The PMSC-ECM product was placed on the spinal cord with the cell-seeded side down. The ECM was sutured into place, and the fetal skin was closed over the ECM. Lost amniotic fluid was replaced with normal saline, and antibiotics (100 mg of gentamicin and 1 million units of penicillin) were added to the amniotic fluid at the time of uterine closure. The fetuses were delivered via cesarean delivery between 142 and 146 days of gestation and, unlike previous studies, the ewes were survived to care for the lambs.

Postnatal Assessment

The SLR was used to assess the motor function of the lambs. The SLR is a standardized and validated system to assess sheep locomotor function [7]. A score of 15 indicates normal locomotor function, 10–14 indicates a mild motor deficit, 5–9 indicates a moderate deficit, and 4 or less indicates a severe deficit [7]. The SLR was assessed on the first and second days of life, then weekly for the first 2 months, and then twice per month until 6 months. Only lambs with an SLR of 15 on the first day of life were selected for the study. If the lamb had a sustained SLR of less than 15 on assessment, it was euthanized prior to 6 months.

A perimortem lumbar MRI was performed on all of the lambs. The MRI images were processed in Horos (Nimble, Annapolis, MD, USA). Spinal angulation was measured in Horos (Nimble, Annapolis, MD, USA) on sagittal imaging. The area of maximum spinal cord compression was determined on axial imaging by identifying where the spinal cord had the most decreased height and the least amount of surrounding cerebral spinal fluid (CSF).

Addition of Physical Therapy

The first 2 lambs were unable to be survived for 6 months, so physical therapy and bracing were added for the subsequent lamb. Spinal X-rays of the third lamb were obtained after birth and monthly.

The physical therapy consisted of 1 h sessions, once to twice daily for 5 days a week with the goal of improving spinal stability through core strengthening and maintaining hind limb muscle strength. Core and pelvic limb muscle strengthening was achieved through a series of therapeutic exercises including tools such as cavaletti poles, cones, wobble boards, and balance discs. To maximize effective therapy, the lamb was also trained to walk and do exercises using a harness. Bracing consisted of wearing a thoracic-lumbar-sacral brace for 8 h per day for 5 days a week with the goal of providing external bracing support. Initially, a L'il Back Bracer (Caerus Corp, Arden Hills, MN, USA) was used. The straps were secured to the lamb taking care to not be too tight and cause abra-

Table 1. Characteristics of the ovine MMC model

	Lamb 1	Lamb 2	Lamb 3
Defect creation			
GA at defect creation, days	75	78	80
Number of fetuses	1	1	2
Horn	Left	Left	Right
Fetal operative time at defect creation, min	30	34	33
Defect creation length, cm	3.2	3.4	3.1
Defect repair			
GA at defect repair, days	98	101	104
Fetal operative time at defect repair, min	54	39	57
Defect repair length, cm	4.5	5	4.4

The lambs had similar GA, operative times, and defect lengths. MMC, myelomeningocele; GA, gestational age.

sions. A custom brace with the same design was used once the lamb outgrew the L'il Back Bracer (Caerus Corp, Arden Hills, MN, USA).

Data Analysis

The motor function outcome and the MRI results were quantitatively compared between the lambs which had no postnatal therapy and the lamb which underwent postnatal physical therapy and bracing.

Results

Three lambs were survived past 48 h. Lambs 1 and 2 did not receive postnatal physical therapy and bracing, while lamb 3 did. The characteristics of the gestational ages at defect creation and repair, fetal operative times, and defect lengths were similar between the 3 lambs (Table 1).

All of the lambs had an SLR of 15 at birth. Lamb 1's SLR declined to 7 at 29 days and lamb 2's SLR declined to 13 at 65 days. This is in contrast to the physical therapy and bracing lamb, lamb 3, who survived 6 months with a persistent SLR of 15 (Fig. 1).

The amount of spinal cord compression varied between the lambs at the time of euthanasia. At the location of maximum spinal cord compression on MRI, lambs 1 and 2 had minimal CSF around the spinal cord, while lamb 3 had much more CSF around the spinal cord (Fig. 2).

The spinal angulation was worse in the lambs who did not receive physical therapy and bracing (lambs 1 and 2) at an earlier timepoint than the lamb who received physical therapy and bracing (lamb 3, Table 2). The spinal angulation of lamb 3 gradually increased over time (Fig. 3).

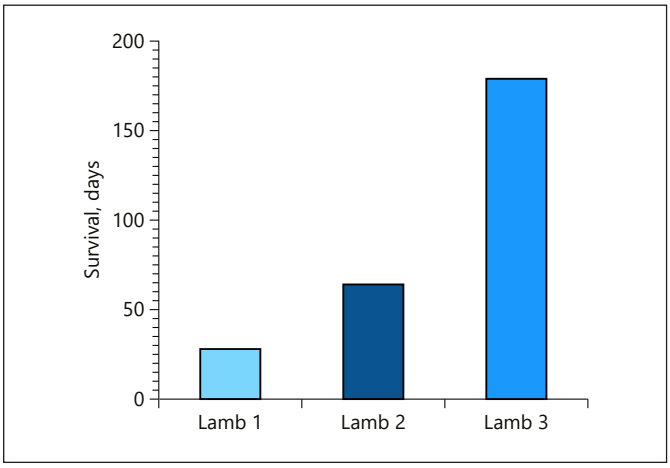

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Fig. 1. Survival of the lambs. Lambs 1 and 2 survived less than 6 months due to a SLR of less than 15. In contrast, lamb 3, who had postnatal physical therapy and bracing, survived the entire 6 months with a consistent SLR of 15. SLR, sheep locomotor rating.

However, the spinal angulation of lamb 3 at 6 months was still less severe than lamb 1 at 1 month and lamb 2 at 2 months (Fig. 4).

Discussion/Conclusion

This pilot study is the first description of surviving lambs from the ovine MMC model treated in utero with PMSCs. When the inherent morbidity of absent paraspinal muscles in a quadruped is removed through physical therapy and bracing, lambs treated in utero with PMSCs can survive for at least 6 months with normal motor func-

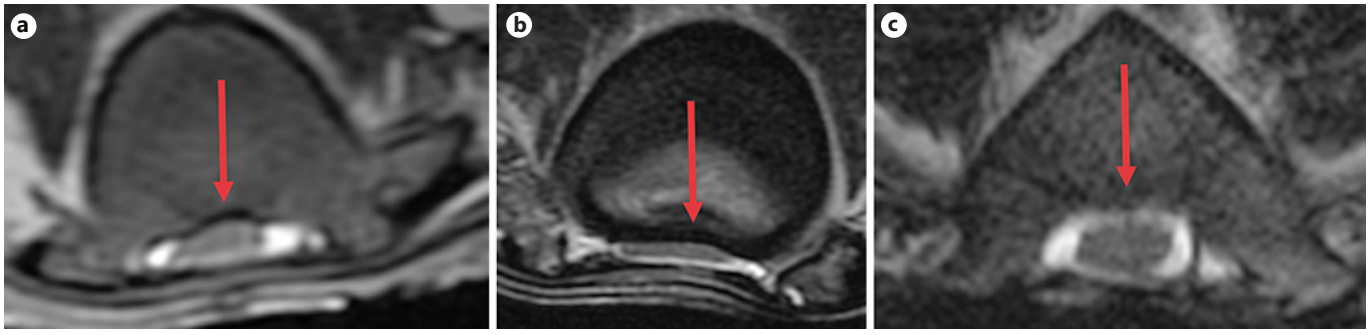

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Fig. 2. Spinal cord compression of lambs 1–3 on MRI at the maximum point of compression. Lamb 1 (**a**) and lamb 2 (**b**) had much less CSF surrounding the spinal cord at the maximum point of compression compared to lamb 3 (**c**). CSF, cerebral spinal fluid.

Fig. 3. Monthly spinal X-rays of lamb 3. The spinal angulation progressively increased each month. mo, months.

tion. The augmented repair with PMSCs in the ovine model is durable for at least 6 months.

In addition to the known limitations of this surgical model, we found that the morbidity of absent lumbar paraspinal muscles and lamina allowed for progressive deformation and angulation of the spine limiting survival [6]. However, with the addition of postnatal physical therapy and bracing, this limitation of the model was minimized. This was evidenced by both decreased spinal angulation of lamb 3 when compared to age-matched time points of lambs 1 and 2 and final angulation of lamb 3 at 6 months which was much less than lamb 1 at 1 month and lamb 2 at 2 months. Both physical therapy and brac-

Table 2. Spinal angulation of the lambs at age-matched time points

	No B/PT	B/PT
1 month	Lamb 1–57°	Lamb 3–19°
2 months	Lamb 2–47°	Lamb 3–33°
6 months	–	Lamb 3–42°

The B/PT lamb had less spinal angulation than the lambs who did not have B/PT. There were no surviving lambs at 6 months who did not have B/PT. B/PT, bracing and physical therapy.

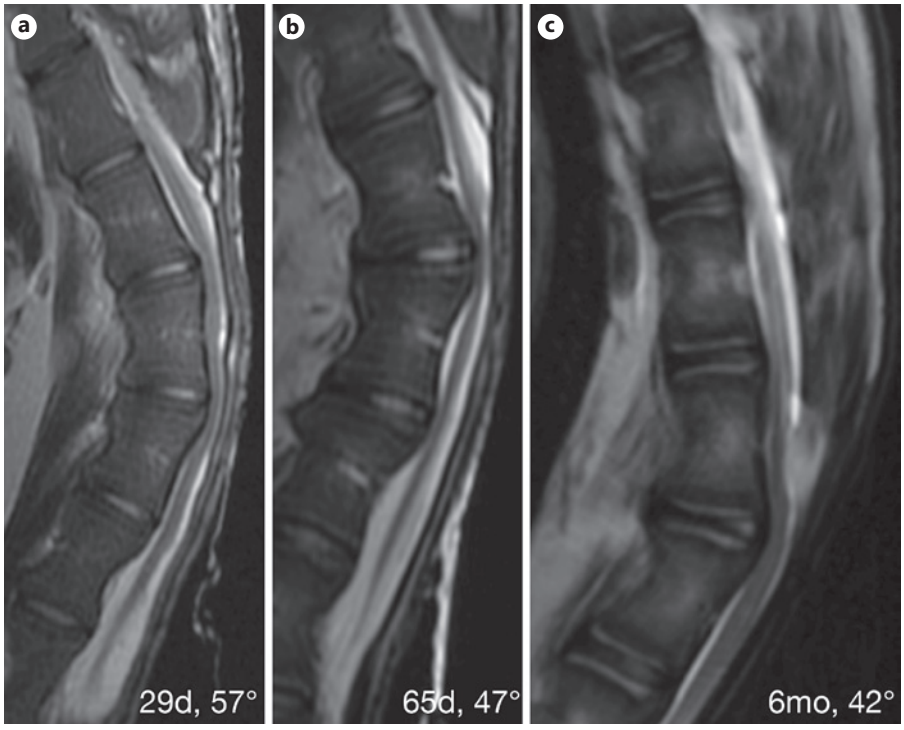

Fig. 4. Spinal angulation of the lambs. Lamb 1 had an angulation of 57° at 29 days (**a**), lamb 2 had an angulation of 47° at 65 days (**b**) and lamb 3 had an angulation of 42° at 6 months (**c**). mo, months; d, days.

ing are used as postnatal treatments for human MMC. Physical therapy can improve a child with MMC's ability to ambulate up to what is expected based on the anatomic level of the defect [11]. Spinal bracing is also used to treat spinal deformities associated with MMC [12]. Physical therapy and bracing are important components of postnatal treatment in the ovine MMC model and in humans with MMC.

The improved motor function outcomes of the ovine model cannot be attributed to physical therapy and bracing alone. In humans, physical therapy maintains the patient's expected functional level based on the anatomic level of the MMC defect but does not typically improve the patient's functional level beyond the anatomic level, as this was shown for the first time in the MOMS trial with prenatal surgery [1, 13]. All of the lambs had normal hind limb motor function after birth, as if there were no MMC defect, which was sustained in the lamb who received postnatal physical therapy and bracing. Thus, the role of physical therapy and bracing in the ovine MMC model is to counteract the inherent musculoskeletal morbidity of the model.

There are several limitations to this study. First, only one lamb was treated postnatally with physical therapy and bracing, limiting the power of the study. Second, all of the lambs did not receive the same cell density which could confound the outcomes. Third, spinal imaging was not obtained at baseline or monthly in lambs 1 and 2, so the progressive angulation of these lambs is inferred. Additionally, the spinal imaging was not obtained with the lambs bearing weight, which would more closely approximate functional angulation while ambulating.

Now that MMC lambs can be survived up to 6 months, there are many new areas of research. Current areas of research include the outcomes of the augmented repair on motor, bowel, and bladder function up to 6 months. These data are ultimately useful to support an Investigational New Drug Application with the Food and Drug Administration. Finally, human clinical trials are being designed to provide fetal MMC patients with the augmented repair.

This is the first description of the survival of lambs from the ovine MMC model past 48 h of life. Fetal lambs with MMC repaired in utero with PMSCs can be survived for at least 6 months with physical therapy and bracing. The rescued motor function from the in utero MMC repair augmented with PMSCs persists for at least 6 months in the ovine model.

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Statement of Ethics

The views expressed in this material are those of the authors and do not reflect the official policy or position of the US Government, the Department of Defense, or the Department of the Air Force. The animals involved in this study were procured, maintained, and used in accordance with the Laboratory Animal Welfare Act of 1966, as amended, and the Guide for the Care and Use of Laboratory Animals, National Research Council. The work reported herein was performed under the University of California Davis Institutional Animal Care and Use Committee protocol number 19676.

Conflict of Interest Statement

There are no conflicts of interest.

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Author Contributions

Study conception and design: K.Y., L.G., J.P., P.K., C.P., A.W., and D.F. Data acquisition: K.Y., L.G., K.H., V.V., B.K., J.B., and C.P. Analysis and data interpretation: K.Y. and L.G. Drafting of the manuscript: K.Y. Critical revision: L.G., J.P., P.K., C.P., S.S., C.T., J.J., A.W., and D.F.

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