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Authors

Stokes, Sarah C

Jackson, Jordan E

Theodorou, Christina M

et al.

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A Novel Model of Fetal Spinal Cord Exposure Allowing for Long-Term Postnatal Survival

Sarah C. Stokes[a], **Jordan E. Jackson**[a], **Christina M. Theodorou**[a], **Christopher D. Pivetti**[b], **Priyadarsini Kumar**[b], **Kaeli J. Yamashiro**[a], **Aijun Wang**[a,b], **Diana L. Farmer**[a,c]

[a]Department of Surgery, University of California-Davis, Sacramento, CA, USA

[b]Department of Biomedical Engineering, University of California-Davis, CA, USA

[c]Shriner's Hospital for Children Northern California, Sacramento, CA, USA

Abstract

Background: The inherent morbidity associated with fetal ovine models of myelomeningocele [MMC] has created challenges for long-term survival of lambs. We aimed to develop a fetal ovine surgical spinal exposure model which could be used to evaluate long-term safety after direct spinal cord application of novel therapeutics for augmentation of *in utero* MMC repair.

Methods: At gestational age [GA] 100–106, fetal lambs underwent surgical intervention. Laminectomy of L5-L6 was performed, dura was removed, and an experimental product was directly applied to the spinal cord. Paraspinal muscles and skin were closed and the fetus was returned to the uterus. Lambs were delivered via Cesarean section at GA 140–142. Lambs were survived for 3 months with regular evaluation of motor function by Sheep Locomotor Rating Scale. Spinal angulation was evaluated by MRI at 2 weeks and 3 months.

Results: Five fetal surgical intervention lambs and seven control lambs who did not undergo surgical intervention were included. All lambs survived to the study endpoint of 3 months. No lambs had motor function abnormalities or increased spinal angulation.

Conclusion: This model allows for long-term survival after fetal spinal cord exposure with product application directly onto the spinal cord.

Keywords

Animal model; Fetal surgery; Myelomeningocele; Spina bifida

Corresponding Author: Sarah C. Stokes, Department of Surgery, University of California, Davis, 2335 Stockton Blvd, Room 5107, Sacramento, CA 95817, USA, Telephone: 916-734-2724, Fax: 916-734-5633, scstokes@ucdavis.edu.

Author Contributions

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

Statement of Ethics

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 21476. 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.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Introduction

Myelomeningocele (MMC), the most severe form of spina bifida, is a birth defect resulting from incomplete closure of the neural tube. Despite a reduction in the rates of MMC with folic acid supplementation, spina bifida remains one of the most common congenital defects, accounting for 3 per 10,000 live births in North America [1]. The MMC defect results in direct exposure of the spinal cord to the intrauterine environment which causes significant neurological morbidity, with varying degrees of lower extremity paralysis, orthopedic disabilities, and bladder, bowel, and sexual dysfunction. The landmark Management of Myelomeningocele Study (MOMS) established fetal surgical repair as the standard of care by demonstrating a reversal of hindbrain herniation, reduction in the need for cerebral spinal fluid shunting and improved motor outcomes for children with MMC who underwent fetal repair [2]. Despite the benefits demonstrated in this trial, only 55% of patients repaired *in utero* were able to walk at 30-month follow up [3]. Therefore, there remains an ongoing unmet clinical need and researchers are currently evaluating the benefits of augmenting the fetal MMC repair with novel therapeutic agents in animal models.

Fetal MMC animal models are designed based on the concept of the two-hit hypothesis of spinal cord damage. According to the two-hit hypothesis, spinal cords that are exposed *in utero* due to primary anomalies in development are secondarily damaged by exposure to amniotic fluid and direct trauma [4]. The ovine fetal MMC model is commonly used due to the large fetal size, long duration of gestation and low rate of preterm labor [5]. In fetal ovine models, MMC defects are created by surgically exposing the spinal cord to the intrauterine environment for a period of time before repair. Given the innate ability of fetuses to heal wounds [6–8], wide removal of skin, subcutaneous tissues, and paraspinal muscles is performed to ensure the defect does not heal during the remainder of gestation, when the spinal cord is intended to accrue damage to emulate naturally occurring human MMC. While this is beneficial for validity of the model, it results in morbidity due to inherent instability of the spinal column, causing increasing spinal angulation as the lamb grows, which results in spinal cord compression and progressive loss of distal neurologic function [9], often requiring humane euthanasia soon after birth. Some models include a myelotomy to induce hindbrain herniation. This allows for evaluation of improvement of hindbrain herniation with repair, but adds to the morbidity of the model [10–12].

Modalities under investigation for augmentation of fetal MMC repair to improve distal neurologic function include biologic materials placed directly on the exposed spinal cord to promote neuronal preservation and cartilage scaffolds for cord protection. While current surgical MMC animal models allow for the evaluation of short-term efficacy of these products, they are limited in allowing researchers to evaluate long-term safety due to the inherent associated morbidity. Other standard animal models to evaluate safety and potential tumorigenicity of biologic products, including NOD/SCID/IL-2rγKO (NSG) mice [13] and transplacental injection of cell for an *in utero* model [14], do not allow for direct application of product to exposed spinal cord *in utero*. The aim of this study was to develop and test a novel surgical spinal cord exposure model which would allow for long-term survival of lambs after placement of a study product directly onto the spinal cord *in utero*. We

hypothesized that this new model would result in the ability to survive lambs long term with normal motor function and no hindbrain herniation and as such would be suitable to test the long-term safety of novel therapeutic products.

Materials and Methods

Fetal ovine spinal cord exposure, repair, and delivery

Animal work was approved by the Institutional Animal Care and Use Committee (IACUC) 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.

At 100–106 days gestation, pregnant ewes were placed under general anesthesia. A midline laparotomy was made. The uterus was exposed, and a hysterotomy was created over a palpable fetus. The back of the fetus was exteriorized. The spinal cord was exposed using microsurgical tools under 3.5x loupe magnification. The fetal skin was incised from L4-L6, and a combination of blunt and sharp dissection was used to expose the lamina. A two-level laminectomy of L5 and L6 was performed. The dorsal portion of the exposed dura was excised. The paraspinal muscles were left intact. Spinal cord exposure is illustrated in Figure 1. Our experimental product was then placed directly on the spinal cord. The fetal paraspinal muscles, subcutaneous tissues and skin were closed in layers. The fetus was returned to the uterus, the lost amniotic fluid volume was replaced with warm saline with the addition of penicillin (1 million units in 1mg) and gentamicin (100mg in 1 ml), and the hysterotomy was closed. The maternal laparotomy was repaired in layers. Fetuses were delivered via survival cesarean section between 140–142 days gestation.

For multi-gestational pregnancies only one fetal lamb per ewe underwent surgical intervention. Fetal lambs who did not undergo *in utero* surgical intervention were also delivered via survival cesarean section and served as normal controls.

Assessment of Long-Term Motor Viability

In order to survive long term, lambs need to be able to ambulate independently. Lamb motor function was evaluated with the validated Sheep Locomotor Rating scale (SLR) [15] at birth, 24 hours, 48 hours and then monthly until the study endpoint. Lambs received a score of 0–15 based on their joint movement, step coordination, and ability to bear weight and clear an obstacle. A score of 15 indicates normal motor function while a score of 0 indicates complete hindlimb paralysis. If the SLR score of an animal dropped below 15 it was euthanized according to the requirements of our IACUC regarding the humane treatment of animals.

Lumbar Spine and Brain Imaging

Magnetic resonance imaging [MRI] of the spine and brain was performed on all of the lambs between birth and 2 weeks and again at 3 months to evaluate for spinal angulation and hindbrain herniation at the MRI Facility for Integrative Neurosciences in Davis, California. Lambs were under general anesthesia during the MRI. The MRI images were processed

in Horos (Nimble, Annapolis, MD). Spinal angulation was measured in Horos (Nimble, Annapolis, MD) on sagittal imaging. Using a previously described technique [9] lines were drawn through the midpoint of lumbar vertebrae on sagittal imaging, intersecting at the L2–3 and L4–5 (Figure 2). These levels were chosen as L2–3 is the previously demonstrated maximum level of angulation in lambs undergoing standard MMC creation and repair [9], and the defects in these study animals were created between L5 and L6. Hindbrain herniation was defined as descent of the cerebellar vermis below the level of the foramen magnum on midsagittal slices [16].

Statistical Analysis

Groups were defined as fetal surgical intervention and controls with no intervention. SLR scores and degree of spinal angulation in each group were compared between groups using the Mann-Whitney U test. P values <0.05 were considered statistically significant. Statistical analysis was conducted using Minitab Version 19.2020.1.0 (Copyright 2020 Minitab, LLC).

Results

Spinal cord exposure and repair was performed in seven fetal lambs. The overall fetal viability was 71%. The five surviving fetal surgical intervention lambs and their seven siblings who did not undergo surgical intervention were included in the study.

The median SLR score of the fetal surgical intervention lambs at all evaluated time points was 15, which corresponds to normal ambulation. The median SLR score of all control lambs at all time points was also 15. No lambs were euthanized for an SLR of less than 15.

Spinal angulation for each individual lamb based on MRIs completed between birth and 2 weeks and MRIs at 3 months is demonstrated in Table 1. There was no significant difference in spinal angulation between fetal surgical intervention lambs and control lambs at the birth to 2 week MRI. Spinal angulation at L2–3 ranged from 2.4–8.5 degrees (median 3.5, IQR 2.4–7.0) in fetal surgical intervention lambs and 1.2–6.7 degrees (median 2.3, IQR 1.7–5.3) in control lambs (p=0.273). Spinal angulation at L4–5 ranged from 1.3–6.9 degrees (median 3.3, IQR 2.3–5.4) in fetal surgical intervention lambs and 1.9–3.8 degrees (median 2.8, IQR 1.9–3.6) in control lambs (p=0.361). Spinal angulation was again evaluated at 3 months. There was again no significant difference between groups. Spinal angulation at L2–3 ranged from 2.5–5.1 degrees (median 3.4, IQR 2.7–4.5) in fetal surgical intervention lambs and from 2.4–3.3 degrees (median 2.9, IQR 3.4–3.2) in control lambs (p=0.144). Spinal angulation at L4–5 ranged from 2.5–5.0 degrees (median 3.5, IQR 3.0–4.7) in fetal surgical intervention lambs and 2.6–5.5 degrees (median 3.8, IQR 2.8–4.7) in control lambs (p=0.855). A representative MRI image of the brain and lumbar spinal cord is demonstrated in Figure 3. There was no evidence of hindbrain herniation for any animals at birth or at 3 months.

Discussion

We have developed a new fetal ovine spinal cord exposure model that allows for long-term survival, consisting of *in utero* laminectomy with spinal cord exposure and application

of an experimental therapeutic product directly to the spinal cord. Lambs that underwent this surgery *in utero* were survived to the planned study endpoint of 3 months. They did not develop worsening motor function, spinal angulation or hindbrain herniation, key limiting factors in long-term survival in large animal MMC models. Critically, this model allows for direct application of experimental products to the spinal cord without incurring the morbidity associated with routinely used models of fetal ovine MMC, which limit the long-term survivability of lambs due to progressive spinal angulation and spinal cord compression.

Routinely used fetal ovine models for surgical creation of MMC result in significant morbidity to the lamb and challenges in long-term survival. These models can be divided into those that induce hindbrain herniation and those that do not. Meuli et al. originally described the creation of an MMC lesion in a fetal lamb model [17]. In this model, fetal lambs were operated on at GA 60 or 75 days, with removal of paraspinal muscles, a laminectomy of L1-L4 and removal of the dura from L1-L4. For animals operated on at GA 75, this model resulted in hindlimb paralysis at birth. However, for lambs operated on at GA 60, this resulted in healed lesions with mild to no hindlimb paralysis for lambs. The ability of the fetal lamb to heal a defect created at GA 60 illustrated the remarkable healing capacity of the fetus. This also limited the model as it shortened the duration of potential time for subsequent spinal cord exposure to the intrauterine environment after defect creation. After establishing a model for defect creation, Meuli et al. repaired ovine MMC fetuses using a latissimus dorsi flap at GA 100 and demonstrated that fetuses repaired in this fashion had near normal hindlimb function at birth [18]. Notably this meant that the spinal cord in this ovine MMC model was exposed to the intrauterine environment for 25 days, while in a human fetus the neural tube is normally closed by GA 30, and the earliest a defect can be repaired is GA 126 resulting in spinal cord exposure to the intrauterine environment for 96 days [19]. This results in spinal cord exposure for 17% of gestation in the ovine fetal model compared to 34% of gestation in the human. Despite these differences from human MMC, the ovine model of fetal MMC repair is considered the gold standard. Other groups have created defects in a similar fashion and repaired the defects with the incorporation of biomatrices[20], amniotic membranes[21], or placental mesenchymal stem cells on extracellular matrices[22–24].

A major cause of morbidity in human patients with MMC is hindbrain herniation [25], and the lack of hindbrain herniation in the Meuli model is a criticism of this model [10]. In our model for long-term survival, we aimed to avoid hindbrain herniation as our primary focus was to create a model of fetal spinal cord exposure that did not result in significant morbidity to the animal and could be used to evaluate long-term safety of experimental products, rather than efficacy. Paek et al. induced hindbrain herniation in a fetal MMC model by adding a myelotomy, an incision of the central canal of the spinal cord, to the defect creation surgery [10]. Hindbrain herniation in unrepaired fetal MMC lambs with a myelotomy has been reported to range from 43% to 100% [10–12]. Models that include hindbrain herniation are still not an exact replica of human MMC as these lambs very rarely develop hydrocephalus [10, 11, 26]. Importantly *in utero* surgical repair has been demonstrated to decrease rates of hindbrain herniation in these animals, as well as improve motor function [10, 11, 18].

In all prior reports of fetal ovine MMC models, animals who underwent *in utero* repair were not able to be consistently survived long term. As described by Vanover et al. [9], the removal of the paraspinal muscles results in instability of the spinal column and can cause spinal angulation, which results in progressive spinal cord compression and loss of motor function as the lamb grows. The majority of ovine studies of *in utero* MMC repair euthanized animals at birth [10] or 48–72 hours after birth [11, 18]. Occasionally studies followed lambs up to one week of age [20]. In our prior attempts at long-term survival, five out of six lambs began developing worsening motor function within one week [27]. We have previously demonstrated the long-term survival to 6 months of one lamb that underwent *in utero* MMC defect creation and repair, which was possible with the addition of physical therapy and bracing orthotic devices [27]. While this is an important advancement, physical therapy is resource intensive, and challenging to provide for multiple lambs simultaneously, which limits the ability of researchers to evaluate long-term outcomes with sufficiently large sample sizes.

We developed this method to enable evaluation of the long-term safety of placental mesenchymal stromal cells seeded onto an extracellular matrix applied to the spinal cord, which we have demonstrated to be efficacious in improving motor outcomes in a fetal ovine MMC model [22–24]. There is a need for this model as other groups develop alternative products to augment the repair. Other notable products currently being evaluated in animals include an umbilical cord patch [28–30] and scaffolds with integrated growth factors [16, 31]. Other groups have demonstrated benefits to MMC defect coverage with intraamniotic injection of bone marrow mesenchymal stem cells [32], amniotic mesenchymal stem cells [33, 34] and microparticles loaded with basic fibroblast growth factor [35]. As fetal treatment of MMC continues to improve motor outcomes in human patients, there is growing interest in the development of a bone or cartilage substitute that can be incorporated into the repair to increase spinal column stability [36]. Bioengineered cartilage for MMC repair has been evaluated *in vitro* [36] and in post-natal repair in rabbits [37]. Evaluation of fetal cartilage engineering *in utero* would allow for further understanding of the impact of the unique fetal environment on engineered tissue. The model described here would facilitate early investigations into bone and cartilage substitutes *in utero* given the small defect size and the high rate of fetal survival.

This model has several limitations. It is a model intended for fetal spinal cord exposure and experimental product application onto the spinal cord. As the spinal cord is exposed and the defect is repaired in the same surgical procedure, there is no time period for spinal cord to accrue damage from the *in utero* environment. Thus, this model does not simulate an MMC defect and cannot be used to evaluate the efficacy of fetal MMC treatments. As seen on MRI, no hindbrain herniation or hydrocephalus results from this model, which again differs from naturally occurring MMC in human patients. However, we noted a critical gap in the available surgical models for fetal MMC repair that prevented the ability to evaluate long-term safety of experimental products and designed this survival model of spinal cord exposure to address this need. It is possible that the application of placental mesenchymal stromal cells seeded on to extracellular matrix improved the motor function in these animals, as we have demonstrated in other models[22–24]. However, even with the incorporation of

placental mesenchymal stromal cells we have only previously been able to survive one lamb long term with our prior fetal surgical model [27].

Conclusion

We have developed a novel surgical model of fetal ovine spinal cord exposure to evaluate the long-term safety of experimental therapeutics used to augment fetal MMC repair, a critical component of preclinical testing. Currently, a major limitation of the current fetal ovine surgical MMC model is the associated morbidity and challenges associated with long-term survival of these lambs. The model described here allows for direct application of an experimental product to the spinal cord of the fetal lamb, and allows for survival of the lambs to at least 3 months. Longer term survival may be possible, and remains to be studied.

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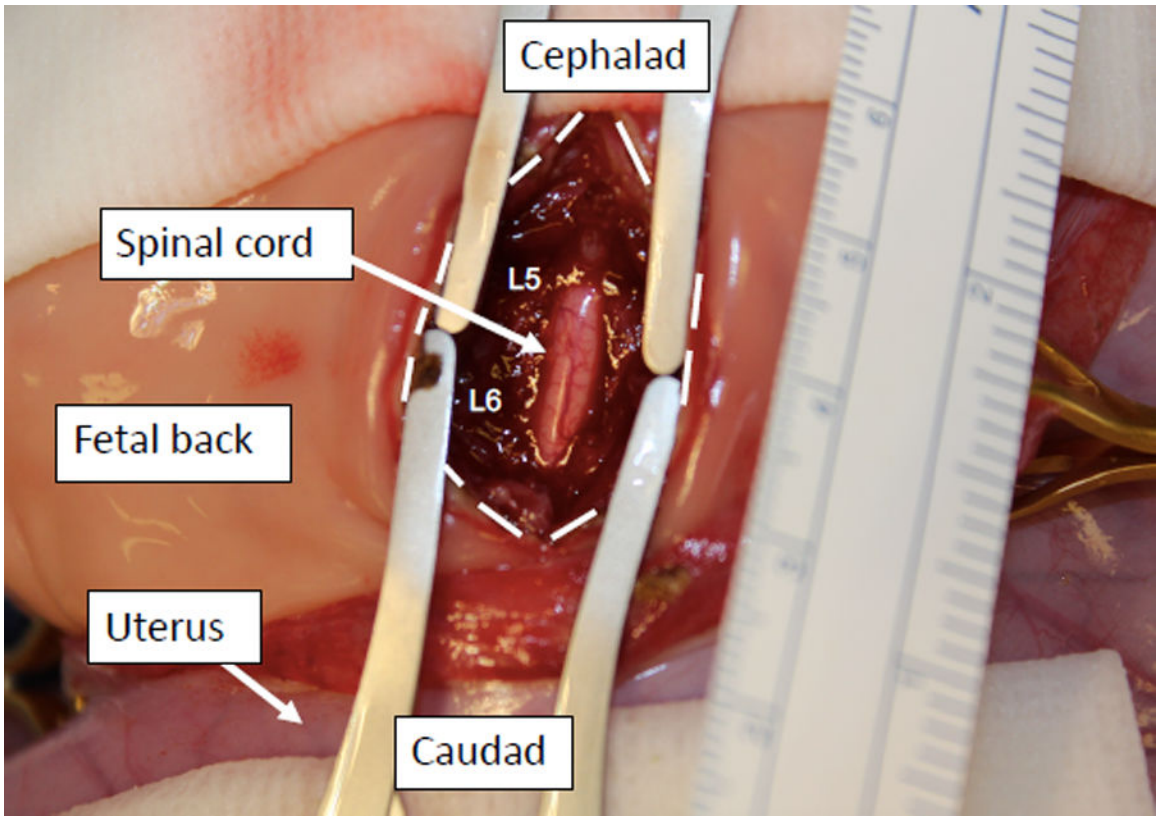

Fig. 1.
Photograph of surgical defect creation. A 2-level laminectomy of L5-L6 was performed and the dorsal portion of the dura was excised.

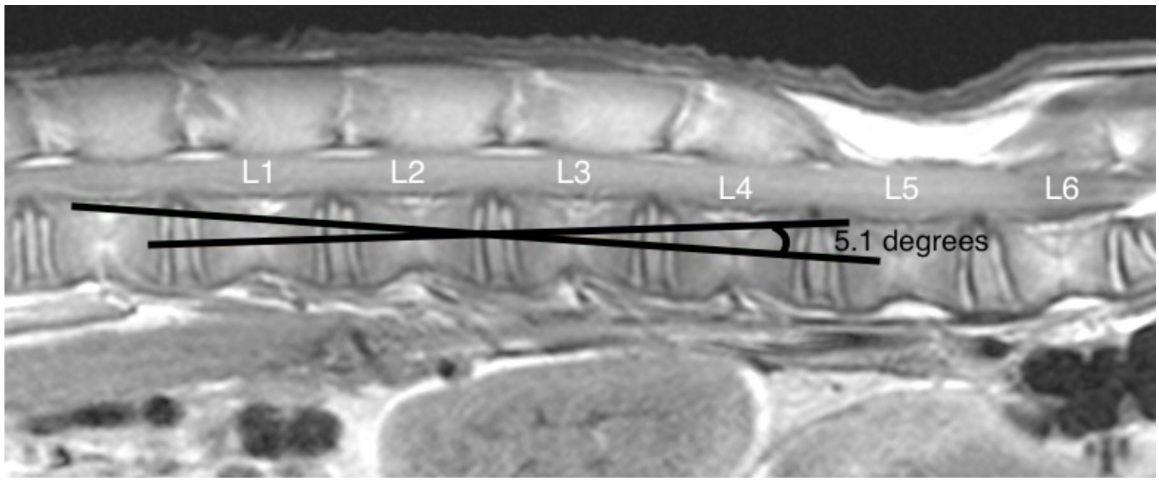

Fig. 2:

Measurement of Lumbar Spine Angulation at L2/L3. Lines drawn through the mid-portion of the lumbar vertebral bodies on mid-sagittal section of an MRI.

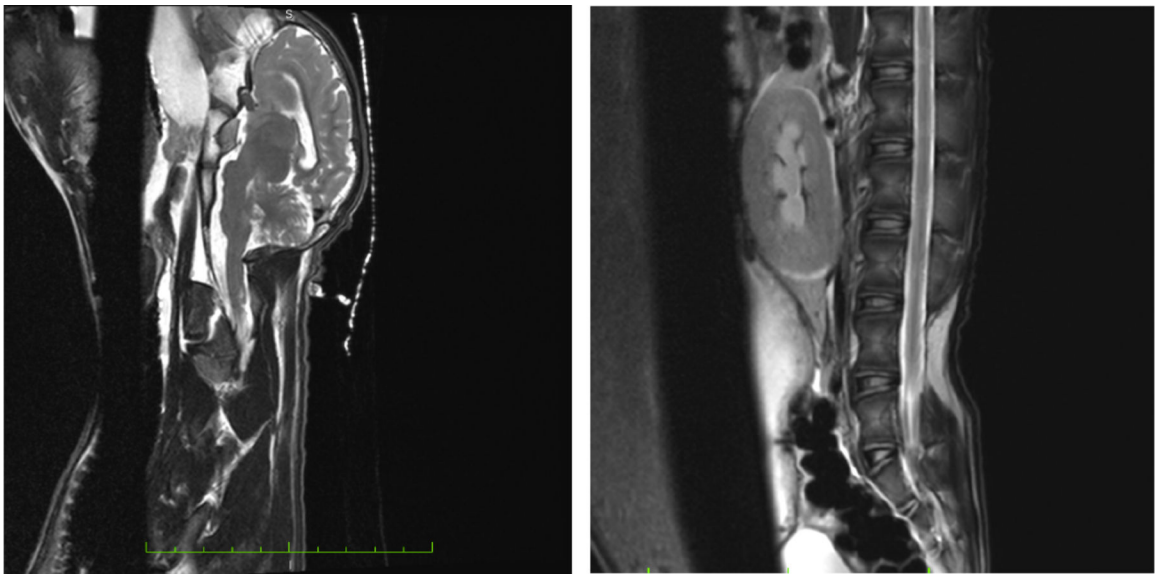

Fig. 3:
MRI of brain [A] and lumbar spine [B] at 3 months of a lamb that underwent surgical intervention

Table 1:

Spinal Angulation at 0 months and 3 months

Sheep ID	Intervention	Birth to 2 weeks old		3 months old	
		Angle L2–3, degrees	Angle L4–5, degrees	Angle L2–3, degrees	Angle L4–5, degrees
1	Intervention	5.49	4.0	5.1	3.5
2	Intervention	2.4	3.3	2.5	4.3
3	Intervention	2.4	6.9	3.9	2.5
4	Intervention	3.5	1.3	3.4	3.4
5	Intervention	8.6	3.3	2.9	5.0
6	Control	1.9	2.1	3.3	4.4
7	Control	6.7	3.5	3.1	4.5
8	Control	1.9	3.0	3.1	2.9
9	Control	1.2	2.5	2.8	5.5
10	Control	2.7	3.8	2.4	2.6
11	Control	4.8	1.5	2.4	3.1