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Neuroprotection is improved by watertightness of fetal spina bifida repair in fetal lamb

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Short title: Watertight fetal spina bifida repair is effective

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Keywords: Neural tube defects, spina bifida aperta, myelomeningocele, fetal surgery, neuroprotection, watertight repair.

CONTRIBUTION

What are the novel findings of this work?

Watertight layered fetal repair of spina bifida is neuroprotective at birth in the fetal lamb model as evidenced by 100% reversal of hindbrain herniation, lower cerebrospinal fluid leakage, better spinal cord neuromotor function, higher brain neuronal density and spinal cord reactive astrogliosis.

What are the clinical implications of this work?

A fetal spina bifida layered repair that achieves watertightness should be adopted clinically regardless of the open or fetoscopic approach to improve neuroprotection.

ABSTRACT

Objectives: the MOMS randomized trial has demonstrated that prenatal spina bifida aperta (SBA) repair via open approach is safe and effective for both mother and fetus, yet half of the infants have incomplete reversal of hindbrain herniation and no improvement in neuromotor function. One contributing factor may be the incompleteness of the neurosurgical repair causing persistent in utero leakage of cerebrospinal fluid (CSF) and exposure of the spinal cord to the amniotic fluid until birth. We aim to investigate the neurostructural and neurofunctional efficacy of the watertightness of prenatal SBA repair.

Methods: a superiority study was conducted in the validated SBA fetal lamb model and powered (n=7 per group). Outcomes of lambs undergoing watertight or non-watertight multilayer repairs through an open approach were compared to those in unrepaired SBA lambs at delivery (term=145 days). At ~75-day fetal lambs underwent standardized induction of lumbar SBA. At ~100 days, they were assigned to one of the three groups and subsequently to either watertight or non-watertight layered repair group based on an intraoperative watertightness test using subcutaneous fluoresceine injection. Finally, at 1-2 postnatal days, we assessed reversal of hindbrain herniation on Magnetic Resonance Imaging (MRI) as primary outcome. Secondary proxies of neuroprotection were CSF leakage at the repair site; hindlimb motor function based on joint movement score, locomotor grade and Motor Evoked Potentials (MEP); neuroprotection score encompassing live birth, hindbrain herniation reversal, absent CSF leakage and joint score ≥9/15; and brain and spinal cord histology and immunohistochemistry. As the watertightness test is not clinically usable, we developed a potential surrogate intraoperative quality score and assessed its relationship with improved outcomes. This four-point scoring system is based on visual assessment of the quality of the skin repair (suture inter-run distance ≤3mm, absence of tear and ischemia).

Results : Compared to unrepaired lambs, watertight repair was neuroprotective in 5/7 lambs (neuroprotection score of 4/4), as evidenced by a 100% reversal of hindbrain herniation on MRI; lower CSF leakage rate (14%); better hindlimb motor function evidenced by higher joint movement score, locomotor grade, and MEP area-under-the-curve and peak-to-peak amplitude; higher neuronal density in the hippocampus and corpus callosum; and higher reactive astrogliosis at the SBA lesion epicenter. Conversely, non-watertight SBA repair did not achieve the same level of neuroprotection (1/7) due to non-significant 86% hindbrain herniation reversal, no motor function improvement, high CSF leakage (43%), low brain neuron count in both regions and low spinal astroglial cell area at the epicenter. Like watertight layered repair, a high quality score (≥2/3) was associated with improved outcomes yet watertightness test and quality score could not be used interchangeably due to results discrepancies.

Conclusions: A watertight layered fetal SBA repair improves brain and spinal cord structure and function in the fetal lamb model. This translational research has important clinical implication and neurosurgical technique that achieves watertightness should be adopted in all fetal centers to improve neuroprotection. Future clinical studies could assess whether the high quality of the repair correlates with clinical outcomes for neuroprotection.

INTRODUCTION

Spina bifida aperta (SBA) is a non-lethal yet progressive congenital malformation of the central nervous system. SBA pathogenesis is explained by two consecutive hits.[1-4] The initial malformation arises when the neural tube fails to close by the 6th week of gestation. This exposes the vulnerable neural elements continuously to direct mechanical and chemical trauma from the amniotic fluid, leading to progressive dysplasia and function loss during gestation. Leakage of cerebrospinal fluid (CSF) through the open ependymal canal leads to a suction gradient[5-7] causing hindbrain herniation[2, 5, 8-10]. After birth, SBA children display various degrees of paraplegia, deformation of spine and limbs, bladder, bowel and sexual dysfunction.[11] They also have variable degrees of neurocognitive impairment[11] as well as Chiari type II malformation[12] and ventriculomegaly that may respectively require posterior fossa decompression and CSF diversion when symptomatic[11, 13].

Given the in utero progressive nature of SBA, prenatal repair was considered first experimentally[3, 14]. Later the Management Of Myelomeningocele Study (MOMS) randomized trial demonstrated that prenatal, as compared to postnatal repair, reduces the shunt rate at 12 months, and increases the chances for children to walk independently at 30 months (45% vs. 24%).[14-16] Fetal neurosurgical repair mimicked the postnatal repair technique consisting of an anatomical “primary” layered repair including at least dura and skin.[14] Arrest of CSF leakage is believed to explain the significant reduction of hindbrain herniation (36% vs. 4% of complete reversal).[14, 17] Prevention of ongoing exposure of the spinal cord and nerves to the amniotic fluid could significantly improves neuromotor function.[16, 17]

The positive effect of the operation seems to be mainly related to the repair watertightness for CSF and amniotic fluid, especially when performed in layers regardless of the open or fetoscopic approach.[18, 19] However, the efficacy of the watertightness of the fetal SBA layered repair has never been assessed neither experimentally[20] nor clinically. We aimed to experimentally determine such efficacy in the validated SBA lamb model.[21] Confirming or refuting this hypothesis bears relevance to the current discussion around modifying neurosurgical open or fetoscopic techniques[22-24] and add relevant evidence to the benefit of fetal surgery for this devastating disease.

METHODS

This study was approved by our university Ethics Committee on Animal Experimentation (P285-2014). It followed the NC3Rs and the ARRIVE guidelines for animal research.[25, 26]

Study design

Given that there was no previous experimental data available and a direct comparison of watertight versus non-watertight layered repair would require very high numbers of animals which is against the above guidelines, indirect comparison[27] was made by comparing each repaired group to unrepaired SBA (historical controls (n=6)[21] plus one animal). We designed a superiority study where primary outcome was complete reversal of hindbrain herniation, measured by Magnetic Resonance Imaging (MRI) at birth.[21] Power calculation was based on the following assumptions: (1) the spontaneous hindbrain reversal rate in unrepaired SBA lambs is 14%[21]; (2) the reversal rate in watertight layered repair should be ≥99%; and (3) two-sided Fisher's exact test with 5% significance level, 80% power. This required seven live born fetuses per group. Experimental animals were respectively assigned to either watertight or non-watertight repair group based on the absence or presence of leakage at the repair site during an intraoperative watertightness test performed immediately after completion of a two-layer SBA repair with patch and skin.

Experimental animals and procedures

Time-dated pregnant Swifter sheep (term: 145 days of gestation) were provided by the university farm. A standardized 4.2x4.2cm L1-6 lumbar SBA defect including myelotomy was induced at mid gestation at ~75 days (Figure 1, Appendix S1).[21]

At ~100 days, this myeloschisis-type defect was repaired in two layers mimicking the MOMS clinical technique[14], i.e. using a dura-fascia replacement patch (DuraGen-Plus) since the model does not allow a more effective multilayer repair using musculofascia flaps[18, 20] covered by native skin (Figure 1, Appendix S1). We also chose the DuraGen matrix because it rapidly provides watertight closure when covered by a watertight layer to prevent CSF leakage while promoting natural dural growth.[28-30] Watertightness is due to initial fibrin clot formation and secondary tissue ingrowth.[28-30] To avoid iatrogenic damage to the spinal cord, we tested the watertightness of the two-layer repair at the end of the repair using diluted fluorescein instilled through a microcatheter inserted under the skin (Figure 1, Appendix S1).[31] Fluorescein was injected at a standardized pressure of 30cm water, which is the highest CSF pressure measured in young children.[32] Leakage was visualized with an ultraviolet flashlight. For animals in the watertight group, additional sutures (range: 0-4) were placed until leakage stopped. For the non-watertight group, the skin was closed but care was taken that there was confirmed leakage on at least one place.

At term ewes were delivered by cesarean section through flank incision under spinal anesthesia.[33] Lambs recovered on the first day and were fed ad libitum.

Outcome measures

All outcomes were standardized and assessed by two independent observers blinded to the allocated experimental condition. These outcomes have been validated and described previously (Appendix S2).[21] On the first day of life, we performed a gross examination of the SBA defect followed by a neurological clinical examination of each lamb[21, 34] mainly to quantify a hindlimb joint movement score

and a locomotor grade with a validated species-specific locomotor rating scale[35]. On day two, general anesthesia was induced and maintained.[21, 33] Lambs first underwent whole-body MRI.[21] Motor Evoked Potentials (MEP)[33] and Somatosensory Evoked Potentials (SEP) were subsequently recorded. Finally the lambs were euthanized and histological samples of the brain, spinal cord from L1-L6, bladder, rectum and hindlimb muscles were harvested and prepared for standard histology and immunohistochemistry.

We also developed a compound neuroprotection score based on a recent clinical systematic review (Appendix S2).[36] We modified this composite five-point scoring system to live birth, complete reversal of hindbrain herniation, absence of CSF leakage and conservation of motor function, evidenced by a joint movement score ≥9/15. Each item was binary and neuroprotection was defined by a maximum score of 4/4.

Intraoperative quality score of skin repair

As the watertightness test is not clinically usable, we also developed a potential surrogate, i.e. an empirical scale for intra-operative visual assessment of the quality of the skin repair. Repairs were scored on a scale from 0 to 3, by scoring three items (as 0 or 1): skin suture inter-run distance ≤3mm, absence of tear and absence of ischemia, based on what was previously used in epithelial wound closure studies.[37] Intraoperative photographs were scored after the experiment by two independent blinded readers and averaged. Lambs were subsequently categorized as either having a “high quality score” (≥2) or “low quality score” (<2).

Statistical methods

We used well-powered and reliable outcome measurements (Appendix S3).[21] Binomial and ordinal variables were expressed as percentage and score, respectively. Chi-square test with Yates' correction was used for comparison due to the three groups. Continuous variables were tested for normal distribution (Appendix S3).[38] Those normally distributed were presented as mean and standard deviation (SD) and compared with one-way ANOVA combined with post hoc Dunnett’s multiple comparison test. Continuous variables not normally distributed were expressed as median and interquartile range (IQR) and compared with the Kruskal-Wallis test combined with post hoc Dunn’s multiple comparison test.

According to the aforementioned guidelines, p values were interpreted based on the assumptions made at the time of the *a priori* power calculation.[39] In other words, results of the primary outcome were considered significant when they were above the predefined threshold of ≥99% for a sample size of 7 per group and with $p<0.05$. To increase statistical robustness, results of the secondary outcomes were powered a posteriori (80% power and 5% significance level) using either the Fisher’s exact test for binary variables or the Sealed Envelope power calculator[40, 41] for continuous variables (www.sealedenvelope.com; superiority study unrepaired vs. watertight two-layer repair). Each secondary outcome was considered significant under three conditions: its p value was <0.05, it was well-powered with a retrospective sample size ≤7 and the primary outcome reached significance.

RESULTS

Models of watertight and non-watertight layered repair of SBA can be established in fetal lambs

Thirty-seven pregnant ewes with a total of 65 fetuses were included (n=14 singletons, n=19 twins, n=3 triplets and n=1 quadruplets) and only one fetus was operated per ewe. In 37 fetuses, SBA was induced at a median of 75 (IQR=2) days. Ten were left unrepaired and used as controls and 7 survived until term. In 27, repair was done at a median age of 102 (2) days (Figure 1). Watertight and non-watertight repair lambs had similar characteristics prenatally and postnatally, 7 in each group surviving until term (Table 1).

Watertight fetal SBA layered repair reverses hindbrain herniation and improves brain histology

Watertight repair lambs presented with complete reversal of hindbrain herniation [7/7 (100%) vs. 1/7 (14%); p=0.001] and absence of brain hemorrhage or ischemia on MRI as compared to unrepaired SBA lambs (Figure 2AB and Table1). In contrast only 6/7 (86%) of non-watertight repair lambs had complete reversal of hindbrain (non-significant; threshold <99%) with no hemorrhage or ischemic changes on MRI (Figure 2AB). In keeping with the macroscopic findings, histology and immunohistochemistry of the brain showed a higher number of neurons in the hippocampus and corpus callosum of watertight repair lambs, without an increased number of apoptotic cells or GFAP (Glial Fibrillary Acidic Protein) positive astrocytes (Figure 2CF). Conversely, a lower number of neurons in the hippocampus and corpus callosum without decreased apoptotic cells or astrocytes was present in the brain of non-watertight repair lambs, no different to the one observed in unrepaired lambs (Figure 2CF).

Watertight fetal SBA layered repair is associated with improvement in spinal cord function and reactive astrogliosis

At the spinal level, all but one watertight repair lamb had complete skin closure without CSF leakage (Figure 3A-C) and tissue coverage of the defect on MRI was thicker than unrepaired lambs (Figure 3DE). They displayed a milder motor deficit with higher joint movement scores and locomotor grades (Figure 4AB). This was paralleled by a higher area-under-the-curve and peak-to-peak amplitude on MEPs (Figure 4EF). Moreover, watertight repair lambs had a larger area of GFAP positive astrocytes (Figure 4CD). Finally, watertight repair was neuroprotective in 5/7 lambs (Table1). One animal had CSF leakage at birth and one with intact skin had a low motoric function score (Table S1).

On the contrary, nearly half of the non-watertight repair animals had CSF leakage at birth (Figure 3AC) despite greater length (Figure 3B) and thicker tissue coverage of the defect on MRI (Figure 3DE). These lambs displayed a motor deficit with low joint movement scores, locomotor grades (Figure 4A-C) and a low area-under-the-curve and amplitude on MEPs, all within the range of the unrepaired (Figure 4EF). On histology the spinal cord presented with a small area of GFAP positive astrocytes similar to unrepaired animals (Figure 4CD). Overall, non-watertight repair was neuroprotective in only 1/7 lambs (Table1 and Table S1).

Statistical interpretation

Power calculation demonstrated that robust conclusions could only be made about brain hemorrhage and ischemia, number of neurons in the hippocampus and corpus callosum, tissue thickness covering the defect, joint-movement score and loco-motor grade, hindlimb MEPs and area of GFAP positive cells in the spinal cord (Figures 2-4, Table1 and Table S2). All watertight and non-watertight layered repair

lambs displayed ventriculomegaly, abnormal diameters of posterior fossa structures, kyphosis and adhesions between the patch and the spinal cord (Table1 and Table S2).

Relation between the intraoperative quality score and the watertightness test

Both watertight layered repair and high quality score were associated with improved outcomes (Table1 and Table S3) as well as neuroprotection since 5/7 animals had a 4/4 neuroprotection score (Table S1). Both tests cannot be used interchangeably since discrepant results were observed for two lambs (Table S1).

DISCUSSION

Principal findings

Prenatal SBA repair has become widely adopted in the last decade after the positive outcome of the MOMS trial.[14, 36] While a number of surgical techniques have been adopted and results are difficult to compare, it is believed that watertightness of the fetal SBA layered repair is critical for the neurological outcome.[22-24] We demonstrated in the validated sheep model that watertight layered fetal SBA repair is neuroprotective, as evidenced by a 100% reversal rate of hindbrain herniation, a low CSF leakage rate at repair site, a better hindlimb motor function, a higher neuronal density in the hippocampus and corpus callosum and a larger area of reactive astrocytes at the lesion epicenter. Conversely, non-watertight repair does not achieve the same level of neuroprotection. This confirms the hypothesis that prevention of continuous exposure of the spinal cord and nerves to chemical trauma from the amniotic fluid significantly improves neuromotor function[16, 17], and arrest of CSF leakage reverses the brain suction gradient causing hindbrain herniation[14, 17].

Results in the context of what is known

Previous studies in the fetal lamb model investigated the functional and morphological effects of fetal layered repair, yet did not look at the effects of its watertightness.[20] In those studies, several outcome measures were used to assess the neuroprotective effect of the repair, including reversal of hindbrain herniation[4, 6], improved lower limb motor function[3, 42, 43], thickness of the ano-rectal sphincters[44], or presence of adhesions around the cord as a proxy for spinal cord tethering[45]. To these we added more sophisticated tests, like MEP analysis[33], quantification of brain neurons and thickness of the detrusor muscle, as well as a functional composite neuroprotection score.

In the MOMS trial, the CSF leakage or dehiscence rate at repair site at birth following open fetal surgery was around 13%.[14] This suggests a persistent open communication in utero prompting local measures after birth and eventually reintervention in 2.6%.[36] Such opening may, next to the postnatal risk of infection, compromise the neuroprotective effect of fetal surgery. Although experts unanimously state that watertight closure is critical[46-51], to our knowledge the functional consequence of non-watertight versus watertight layered repair has never been quantified, neither has a clinically acceptable method being proposed to test the watertightness of a repair intra-operatively.[48] An indirect test based on the presence or absence of a “bulging patch” was suggested by Kohl.[46] Next to the demonstration of “bulging” by accumulating CSF at the end of the procedure, absence of CSF leakage on provocation, i.e. when compressing the bulging with an instrument, should also be demonstrated.[46] We think the bulging test is clinically impractical for the following reason. The CSF is produced at a rate of maximum 0.37mL/min, independent of the size of a fetus or an infant.[52-55] Under the assumption that a bulging of a 4x3cm patch would be reliably confirmed, e.g. by elevation by 0.5cm, one would need to wait for 11.5min following skin closure. This calculation is based on the volume of CSF needed to fill the distance between untethered cord in the spinal canal and the top of such a bulging patch, roughly two thirds of a cm. This can be represented by a volume of two thirds of ellipsoid of 4x3x1cm in diameter. Finally, this test would only be feasible after a skin patch repair, which is practiced in approximately 20% of cases.[56, 57]

Clinical implications

In an attempt to find a clinically reliable CSF leakage test at the time of the operation, we investigated the use of a intraoperative quality score as a translational surrogate for the watertightness test. Both test and score were associated with the level of neuroprotection, although they cannot be interchanged because abnormal test and score did not perfectly identify the same animals. If a skin repair would appear to be inappropriate with inter-run distance >3mm, we would add additional suture. In case of insufficient tissue or tear or ischemia we would consider a skin substitute or skin flap – yet not relaxing incisions – to ensure watertightness.[14, 58-60] It would be very interesting to reassess clinical series for the relationship between repair watertightness - defined by an inter-run distance ≤3mm and absence of tear or ischemia - and hindbrain herniation reversal as well as preservation of motor function. Prospectively we would suggest to systematically report on the quality of the repair at the time of surgery, take a picture and correlate it to the clinical outcomes for neuroprotection.

Strengths and Limitations

Our study also has a number of strengths. It followed the international guidelines for animal research and for validation of animal disease models[25, 61, 62] and for improving statistical interpretation and reporting[39]. The experiment was sufficiently powered to determine efficacy. We used a validated animal model for SBA resulting in a complete and homogeneous phenotype.[21] Outcome measures were obtained by experienced observers whenever possible blinded to the treatment groups and only reliable measurements were used.

We are aware of a number of study limitations. One major generic criticism is the nature of surgical models for SBA. The lesion is induced late in gestation hence unable to replicate the primary embryonic defect of the “first” hit, limiting the effects to “second” hit. This adds to other generic objections to the sheep model having different placental structure and unfused fetal membranes.[63] Second, not all lambs in the watertight groups survived until MEP/SEP recordings, leading to incomplete data in those. Nevertheless, surviving lambs were the ones with the worst joint scores and motor grades. We have previously shown that the MEPs amplitude are correlated with the joint score and locomotor grade[21], so we do not think we overestimated the improvement observed. Finally, this collagen patch induced in all animals in both groups adhesions to the spinal cord. Clinically, this could induce postnatal spinal cord tethering with or without intradural inclusion cyst associated with functional loss.[18, 58, 64, 65] Such collagen patch should therefore be avoided or not be directly applied to the spinal cord.

CONCLUSION

In lambs, watertight two-layer fetal SBA repair is most effective in reversing hindbrain herniation and preserving brain neurons and peripheral neuromotor function. Non-watertight repair has less favorable neuroprotective effects. These translational research findings have direct clinical implications and neurosurgical technique that achieves watertightness should be adopted to improve neuroprotection. They are also of paramount importance to advance the care of fetuses affected by SBA and guide the advancement of fetal surgical techniques.

ADDITIONAL INFORMATION

Data availability

The data that support the findings of this study as well as the custom-made MATLAB algorithm are available from the corresponding authors, upon reasonable request.

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Author Contributions

LJ, FVC and JD designed the entire study. LJ performed all surgeries and animal preparations with help of JP, ED, AC Engels and S. Pranpanus. MGMCMC and SDV performed the clinical evaluation of all lambs. MA and PP analyzed the MRI data. M. Deprez and AK. helped recording and interpreting MEPs. The MATLAB algorithm for MEP analysis was developed by AK. AK, MD, and LJ performed MEP analysis using the custom-made algorithm. Histological analysis was done by LJ, JVDM, JP, ED and ER. LJ and JVDM performed statistical analysis of the data. LJ wrote the manuscript with JD. All co-authors approved the study design, revised and approved the final manuscript.

Conflicts of Interests

The authors declare no competing financial interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Paper presentation information

Our translational research was presented at the SMFM 2021 Virtual 41st Annual Meeting from January 25 to 30 and received an award for best scientific oral presentation.

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LEGENGS OF FIGURES AND TABLES

Figure 1 – Experiment timeline and study groups in the SBA fetal lamb model.
At ~75 days of gestation, surgical induction of SBA with myelotomy (arrow).
At ~100 days, absence of SBA repair (unrepaired SBA group) or SBA non-watertight (leakage of injected fluoresceine via a subcutaneous microcatheter) or watertight (absence of leakage) repair.
The surgical repair steps are: (1) Circumferential incision medially to the junction line between the abnormal and normal skin, circumferential resection of the junction line without touching the normal skin, circumferential skin undermining for ≥2cm and circumferential incision around the placode simulating untethering; (2) first layer of the repair using a bovine collagen dural patch (DuraGen Plus matrix, Integra Life Sciences, Plainsboro, NJ, USA) trimmed to cover the placode with an additional 1cm margin, and sutured at the four corners; (3) second layer of native skin closed with two half running sutures. *Artistic drawings of fetal lamb in the uterine cavity by Christine Bouguet-Joyeux and in transversal plane by Myrthe Boymans (www.myrtheboymans.nl) for and copyright by UZ Leuven, Belgium. (SBA, spina bifida aperta)*

Figure 2 – Brain findings at birth. Comparison between unrepaired SBA, nWT and WT fetal repair groups reporting p value of SBA vs. nWT and SBA vs. WT repair. **(A)** Complete reversal of hindbrain herniation assessed on **(B)** MRI mid-sagittal images (14% (1/7) vs. 86% (6/7) [p>0.05] or vs. 100% (7/7) [p=0.001**]) where the stars pinpoint the hindbrain and the bars the foramen magnum; **(C)** Densities of Nissl-stained neurons in six clinically relevant regions of interest, significantly different in the hippocampus (1023 ± 170 vs. 1183 ± 118 [p=0.246] or vs. 1445 ± 216 [p=0.004**]); and the corpus callosum (479 ± 141 vs. 661 ± 79 [p=0.085] or vs. 712 ± 178 [p=0.038*]); **(D)** Representative fields from Nissl-stained brain slides (arrows showing neurons); **(E)** Densities of NeuN, Caspase and GFAP positive cells in two main brain regions: in the hippocampus with NeuN density of 711 ± 102 vs. 812 ± 37 [p=0.455] or vs. 1034 ± 189 [p=0.010*], Caspase density of 1.0 (8.3) vs. 2.5 (27.8) [p=1.000] or vs. 5.5 (2.3) [p=0.430] and GFAP density of 293 ± 119 vs. 180 ± 71 [p=0.179] or vs. 173 ± 66 [p=0.152]; and in the corpus callosum with NeuN density of 279 ± 58 vs. 326 ± 77 [p=0.701] or vs. 561 ± 126 [p=0.004**], Caspase density of 1.5 (1.8) vs. 0.0 (75.0) [p=1.000] or vs. 4.5 (12.0) [p=0.719] and GFAP density of 275 ± 129 vs. 241 ± 101 [p=0.877] or vs. 149 ± 108 [p=0.248]. **(F)** Representative fields from immunostained brain slides (arrows showing NeuN positive green cells with few Caspase-3 positive red cells). *Abbreviations: SBA, spina bifida aperta; MRI, magnetic resonance imaging; HH, hindbrain herniation; HipC, hippocampus; CA1, Cornu Ammonis 1, first of the four regions of the hippocampus; CC, corpus callosum; GFAP, glial fibrillary acidic protein. Significance: * 0.01<p≤0.05; ** 0.001<p≤0.01.*

Figure 3 - Spinal cord structural findings at birth. Comparison between unrepaired SBA, nWT and WT fetal repair groups reporting p value of SBA vs. nWT and SBA vs. WT repair. **(A)** Pictures of unrepaired SBA and nWT and WT fetal repair lambs; **(B)** Size of skin defect in length (7.3 ± 1.4 mm vs. 14.1 ± 3.0 [p<0.001****] or vs. 9.3 ± 1.7 [p=0.101]) and width (3.0 (4.0) mm vs. 0.0 (0.5) [p=0.003**] or vs. 0.0 (0.0) [p<0.001***]); **(C)** Absence of CSF leakage at repair site (0% (0/7) vs. 57% (4/7)

[p=0.076] or vs. 86% (6/7) [p=0.007**]); **(D)** Thickness of tissue covering the defect (0.0 (1.1) mm vs. 2.0 (1.1) [p=0.010*] or vs. 1.7 (1.5) [p=0.006**]) on **(E)** sagittal MRI images (arrows showing the absence or presence of tissue): *Abbreviations: SBA, spina bifida aperta, nWT, non-watertight repair; WT, watertight repair; CSF, cerebrospinal fluid. Significance: * 0.01<p≤0.05; ** 0.001<p≤0.01; *** 0.0001<p≤0.0001; **** p≤0.0001.*

Figure 4 – Spinal cord and nerve function at birth. Comparison between unrepaired SBA, nWT and WT fetal repair groups reporting p value of SBA vs. nWT and SBA vs. WT repair.
(A) Neurological examination of the hindlimbs of the lambs; **(B)** Locomotor rating by joint movement score (3.2 ± 2.2 vs. 7.0 ± 4.4 [p=0.130] or vs. 9.3 ± 4.1 [p=0.017*]) and locomotor grade (1.4 ± 0.7 vs. 2.8 ± 1.7 [p=0.208] or vs. 4.2 ± 2.2 [p=0.012*]); **(C)** Area of GFAP, b3T and MBP positive cells on immunohistochemistry slides of the spinal cord; **(D)** images of significant results from the GFAP positive astrocytes (7.03 ± 3.85 x 106 pixels vs. 6.11 ± 1.92 [p=0.977] or vs. 19.52 ± 16.26 [p=0.049*]; **(E)** Area-Under-the-Curve (3.5 ± 3.5 vs. 5.1 ± 4.3 [p=0.765] or vs. 11.8 ± 10.3 [p=0.020*]) and Peak-to-Peak amplitude (0.08 ± 0.07 µvolts vs. 0.13 ± 0.11 [p=0.462] or vs. 0.24 ± 0.17 [p=0.025*]) as determined by quantitative Motor Evoked Potentials (MEP) of the hindlimbs; **(F)** Display of MEP recordings for each group. *Abbreviations: SBA, spina bifida aperta, nWT, non-watertight repair; WT, watertight repair; GFAP, glial fibrillary acidic protein; b3T, beta-3-tubulin; MBP, myelin binding protein.*
*Significance: * 0.01<p≤0.05; ** 0.001<p≤0.01; *** 0.0001<p≤0.0001; **** p≤0.0001.*

Appendix S1 - Experimental animals and procedures

Appendix S2 - Outcome measures

Appendix S3 - Statistical methods

Table 1 – Characteristics and outcomes of unrepaired vs. non-watertight or watertight repaired SBA. *Abbreviations: SBA, spina bifida aperta; WT, watertight; nWT, non WT; MRI, magnetic resonance imaging; NA, non-applicable. Significance: * 0.01<p≤0.05; ** 0.001<p≤0.01; *** 0.0001<p≤0.0001; **** p≤0.0001.*

Groups	**Unrepaired SBA**	**nWT fetal repair**	**P value**	**WT fetal repair**	**P value**
Characteristics					
Prenatally					
Gestational Age at induction (days)	N=10 75 (1)	N=19 75 (2)	0.122	N=8 74 (0)	0.770
Gestational Age at repair (days)	NA	N=11 103 (2)	NA	N=7 102 (0)	0.364
Postnatally	N=7	N=7		N=7	
Survival at birth	70% (7/10)	64% (7/11)	0.877	100% (7/7)	0.342
Birth weight (Kg)	3.5 ± 0.8	3.9 ± 0.8	0.598	3.3 ± 0.8	0.820
Primary Outcome					
Reversal of HH on MRI	14% (1/7)	86% (6/7)	>0.05	100% (7/7)	**0.001****
Secondary outcomes					
Brain					
MRI					
Hemorrhage	0% (0/7)	0% (0/7)	1.000	0% (0/7)	1.000
Ischemia	0% (0/7)	0% (0/7)	1.000	14% (1/7)	1.000
Spinal cord					
Histology					
Adhesions between patch & spinal cord	NA	100% (7/7)	NA	100% (7/7)	NA
Overall neuroprotection					
Neuroprotection (score 4/4)	0% (0/7)	14% (1/7)	1.000	**71% (5/7)**	0.026*

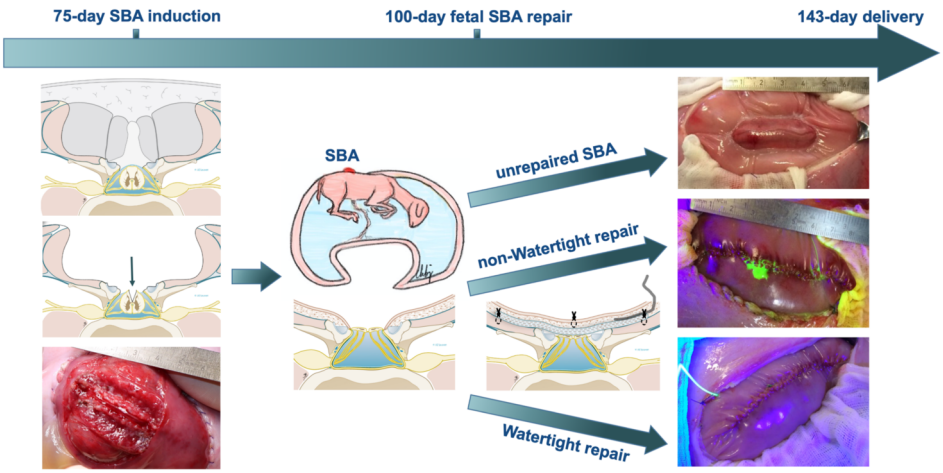

Fig 1-experiment timeline.tiff

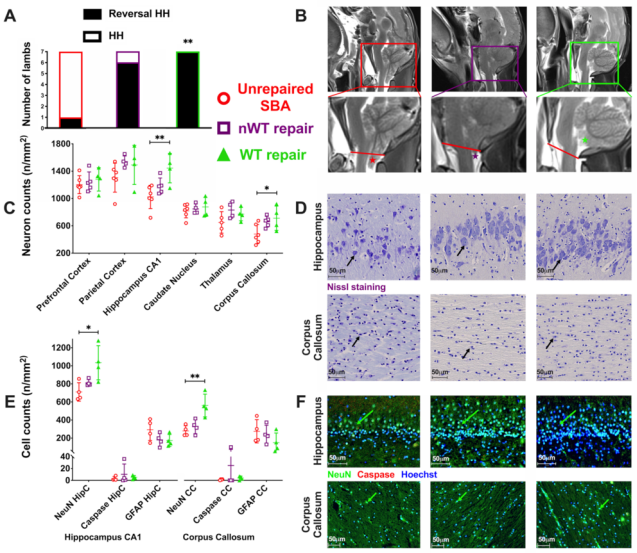

Fig 2-Brain structure.tiff

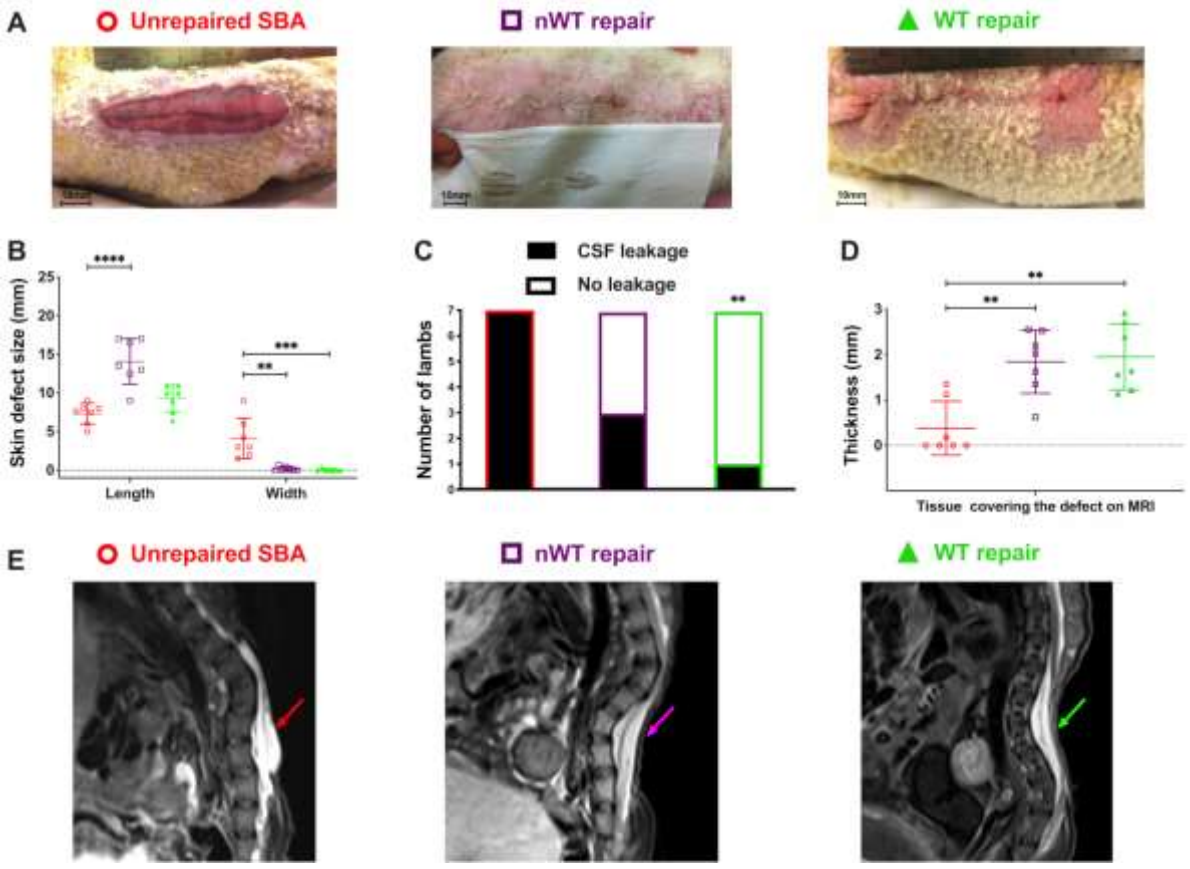

Fig 3-SC structure.tiff

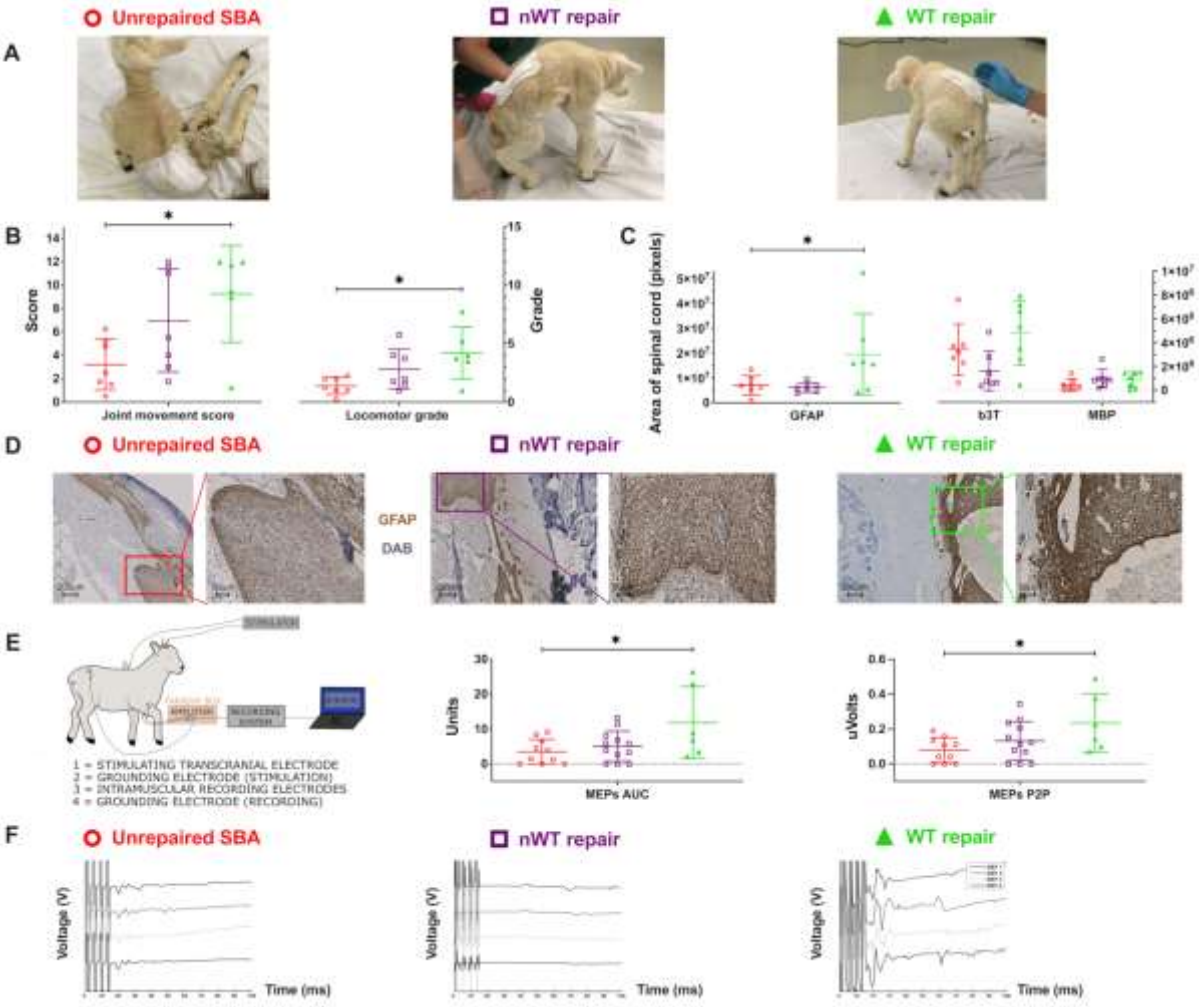

Fig 4-SC motor function.tiff