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## In utero repair of myelomeningocele with autologous amniotic membrane in the fetal lamb model

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### ABSTRACT

**Background:** Despite advances in prenatal repair, myelomeningocele (MMC) still produces devastating neurologic deficits. The amniotic membranes (AM) are a biologically active tissue that has been used anecdotally for human fetal MMC repair. This study evaluated the use of autologous AM compared to skin closure in an established fetal MMC model.

**Methods:** Seven fetal lambs underwent surgical creation of MMC at gestational age of 75 days followed by *in utero* repair at gestational age of 100 days. Lambs were repaired with an autologous AM patch followed by skin closure (n = 4) or skin closure alone (n = 3). Gross necropsy and histopathology of the spinal cords were performed at term to assess neuronal preservation at the lesion.

**Results:** An increase in preserved motor neurons and a larger area of spinal cord tissue were seen in AM-repaired lambs, as was decreased wound healing of the overlying skin. Loss of nearly all spinal cord tissue with limited motor neuron preservation was seen in skin only-repaired lambs.

**Conclusions:** AM-repaired lambs showed increased protection of spinal cord tissue compared to skin only-repaired lambs, but the overlying skin failed to close in AM-repaired lambs. These results suggest a potential role for AM in fetal MMC repair that warrants further study.

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Myelomeningocele, commonly known as spina bifida, is a non-fatal, but devastating congenital anomaly that leaves afflicted children with lifelong lower extremity paralysis, skeletal deformities, urinary and fecal incontinence, sexual dysfunction, and cognitive disabilities. Myelomeningocele (MMC) results from incomplete closure of the neural tube during development, which leaves the spinal cord partially exposed and vulnerable to damage from intrauterine trauma. MMC is one of the world's most common birth defects. Approximately 1500 children in the US alone are born with this life-altering disease every year; this is equivalent to six children born per day with MMC [1].

For centuries, there has been little hope for treating this permanent birth defect. In 2011, however, the results of our NIH/NICHD Management of Myelomeningocele Study (MOMS), which compared prenatal and postnatal closure of the defect using a skin closure repair method in a randomized controlled trial, demonstrated that prenatal repair was safe, improved

associated hindbrain abnormalities including the Chiari malformation and partially improved paralysis in some patients [2]. This study suggested for the first time that paralysis can be improved in this disease. Despite the promise of these findings, the majority of patients continued to suffer from disabling spinal cord defects.

Anecdotal experience of one patient repaired prenatally using a fetal amniotic membrane (AM) patch suggested that such a patch might improve outcomes. The AM is located between the maternal uterus and the fetus. The AM completely surrounds the fetus, and is composed of a fused inner fetal layer, the amnion, and an outer uterine layer, the chorion [3,4]. These membranes are derived from cells of the developing fetus and play a key role in fetal development.

The AM has been shown to have anti-inflammatory effects and to promote epithelialization [3,4]. These properties have led to its use in diverse surgical settings, including ophthalmology and wound care [3–10]. Based on anecdotal experience and extensive literature supporting the anti-inflammatory and epithelialization-promoting properties of AM, we hypothesized that the use of autologous AM during *in utero* MMC repair would provide improved protection to the spinal cord. The goal of this study was to evaluate the use of autologous AM in comparison to skin closure in an established fetal model of MMC.

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## 1. Materials and methods

Experimental protocols were approved by the Institutional Animal Care and Use Committees. All animal care was in compliance with the Guide for the Care and Use of Laboratory Animals, and all animal facilities are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International [11].

Six time-mated, pregnant ewes arrived at the housing facility one week prior to the first surgical intervention. The ewes had free access to food and water except for the 24-hour period directly preceding surgery. The first operation was performed at approximately 75 days of gestation, at which time each ewe underwent survival laparotomy and hysterotomy followed by creation of the MMC defect. As described previously, the MMC defect was surgically created by exposing the spinal cord and removing the dura of each fetal lamb [12,13]. A second survival laparotomy and hysterotomy were performed at approximately 100 days gestation. At this time, fetal lambs underwent repair of the MMC defect with either skin closure alone or with the introduction of an autologous AM patch followed by skin closure. The AM patch, harvested from the placenta at the time of the second hysterotomy, consisted of the fused amnion and chorion. The AM patch was placed chorion side down over the exposed spinal cord and secured with interrupted 6–0 absorbable sutures.

Lambs were delivered via terminal cesarean section around gestational age (GA) 135 days in order to optimize histopathologic analysis or via spontaneous vaginal delivery at term (GA-145). All lambs were sacrificed and perfused with lidocaine and heparin, followed by 1 L of 0.9% NaCl and 2 L of 4% paraformaldehyde. Lamb brains and spinal cords were harvested for pathological analysis and grossly inspected. Cross-sections of the lumbar segments of the spinal cords were stained for histopathological analysis using hematoxylin and eosin, cresyl violet, and Masson's trichrome. Slides were examined for the amount of neural tissue in the spinal cord as well as the presence and quantity of motor neurons at the level of the lesion. Spinal cord tissue area and motor neuron counts were analyzed in 3 cross-sections per animal that were taken at 10 mm intervals that spanned the entire length of the midsection of the lesion, L3–L5 respectively. Spinal cord tissue area was selected and calculated using ImageJ, and did not include dorsal root ganglia if present in the sample. Motor neuron counts were performed by counting cells that were between 30 and 70  $\mu\text{m}$  in diameter and had a discernable nucleus as previously described by Gensel, et al [14]. Statistical analysis for both spinal cord tissue area and motor neuron counts was performed using unpaired *t*-tests.

## 2. Results

Among the six ewes, there were a total of eleven fetal lambs. Four fetal lambs underwent repair with AM followed by skin closure; three lambs underwent standard skin closure repair. Two lambs were left with their defect unrepaired and two lambs were left without a surgically created defect to serve as negative and positive controls respectively. Surgical creation and repair of the MMC defect occurred without immediate complication in all ewes. The MMC lesions spanned spinal cord levels L1–L5. Two ewes were unable to carry the pregnancy to term. One ewe, pregnant with one AM-repaired lamb, one skin only-repaired lamb, and one unrepaired lamb, required euthanization at GA-112 secondary to peritonitis; the fetal tissue was saved, but not perfused. The second ewe, pregnant with one AM-repaired lamb and one unrepaired lamb, aborted the pregnancy at GA-134 (the GA at which a cesarean section would normally be performed). The fetal tissue was saved and perfused. The remaining four ewes carried the pregnancy to term or to planned cesarean delivery with no adverse events. Two lambs (1 AM, 1 skin only-repair) were delivered via terminal cesarean section. The two negative controls were delivered via cesarean section at GA-137 and GA-133.

Two lambs (1 AM, 1 skin only-repair) were delivered via spontaneous vaginal delivery. The brain and spinal cord from all lambs were harvested for pathological analysis as described above. Gross observation of all fetal specimens revealed no major abnormalities other than the surgically created MMC defect. The two negative controls demonstrated no abnormalities and a grossly normal spinal cord. The skin overlying the surgical defect on the three lambs repaired with skin closure remained intact (Fig. 1A), while the skin of three of the four lambs repaired with AM failed to heal (Fig. 1B). This wound-healing deficit was specific only to AM-repaired lambs and has not been seen in any prior fetal MMC models.

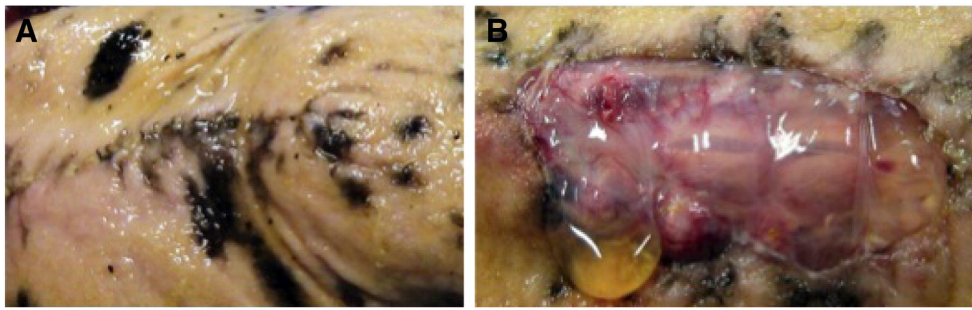
Gross inspection of the spinal cords revealed several differences among the treatment groups. As expected, no abnormalities were seen in the spinal cords of the negative control group. In the unrepaired, positive control group, the spinal cords were extremely thin at the level of the lesion, and scar tissue and fibrinous exudate were the predominant features. The skin only-repaired spinal cords showed only a small improvement over the unrepaired positive controls, but remained flattened; additionally, there was a significant amount of scar tissue noted between the skin and the spinal cord. When examining the AM patch repairs, the patch remained intact on all spinal cords, but had not incorporated into the native tissue. Masson's trichrome staining revealed that the patch consisted of collagen material without fibrosis. Cresyl violet staining revealed the presence of neural tissue in the spinal cord along with the presence of motor neurons as further evidence of preservation of the native spinal cord tissue. The spinal cord appeared substantially preserved and demonstrated fewer adhesions compared to the unrepaired positive controls and the skin only-repair groups (Fig. 2A).

The spinal cord tissue area and the number of motor neurons present at the MMC lesion site were calculated for each spinal cord specimen, and all lambs were included in the analysis. The average area of spinal cord tissue in the AM-repaired lambs was 7.30  $\text{mm}^2$  compared to 1.87  $\text{mm}^2$  in the skin repaired lambs. This difference was statistically significant with a *P* value of 0.05 (Fig. 2B). Unrepaired lambs demonstrated a loss of nearly all spinal cord tissue with a tissue area of 0.75  $\text{mm}^2$ . Analysis of the quantity of motor neurons revealed a significant increase in preserved motor neurons in the AM-repaired group at 30 motor neurons in comparison to 9 motor neurons in skin only-repaired lambs (*p* = 0.02). No preserved motor neurons were seen in the unrepaired lambs (Fig. 3).

## 3. Discussion

This study investigated the use of an autologous AM patch during prenatal repair of MMC in an established fetal lamb model. Pathologic and histological analysis demonstrated a significant increase in both the spinal cord tissue area and the number of preserved motor neurons in lambs repaired with AM compared to lambs repaired with only skin closure. In addition, AM-repaired lambs exhibited fewer spinal cord adhesions, but they also displayed significant wound-healing abnormalities. These results support the existing literature regarding the anti-inflammatory and protective effects of AM, but also underscore a need for caution owing to the marked inhibition of skin wound healing that could result in failure to achieve the previously demonstrated advances in treating the Chiari malformation by failing to prevent CSF (cerebrospinal fluid) leakage.

Although the exact mechanism by which the AM mediates the improvements described above is unknown, several properties of the AM may contribute to this functionality. First, the AM may serve as a protective physical barrier. The importance of spinal cord protection in MMC repair is based on evidence that the disease results from both abnormal neural tube development and subsequent spinal cord damage during gestation and delivery, the latter of which can be ameliorated with increased protection *in utero* [13,15]. The increased preservation of spinal cord tissue in AM-repaired lambs compared to

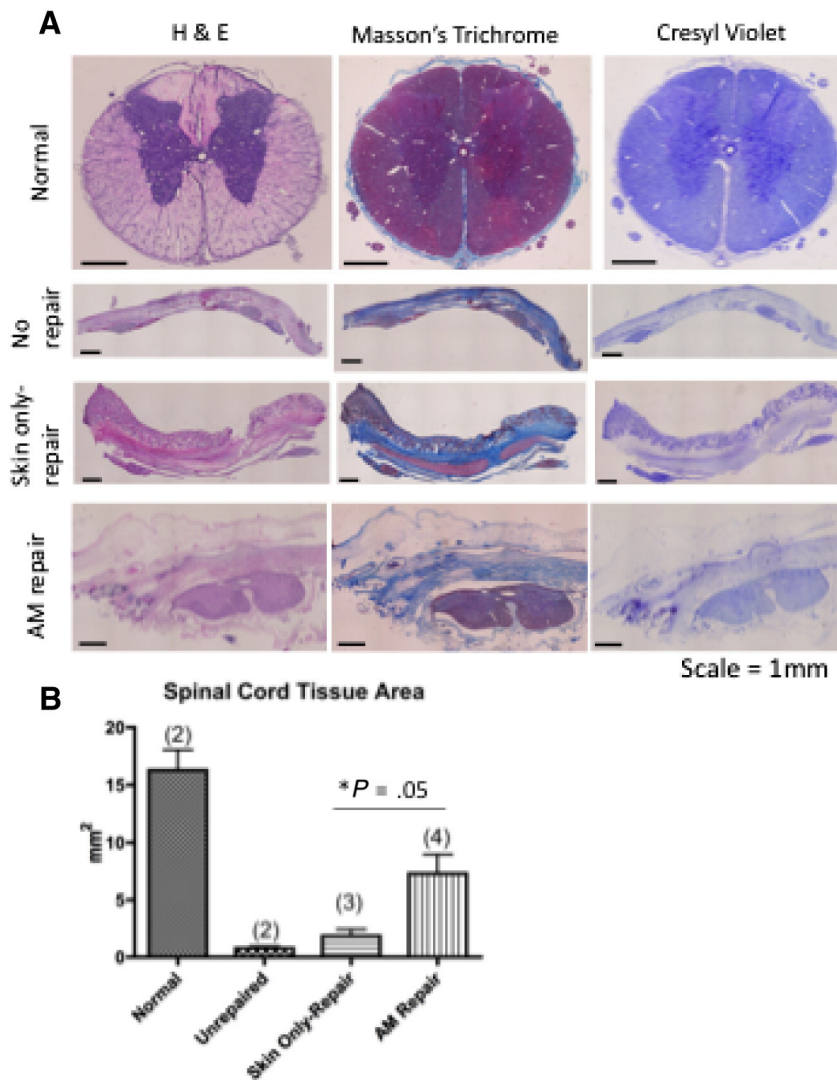


**Fig. 1.** Gross pathology images of the MMC lesion site after cesarean birth at GA-133 for skin only-repaired (A) and AM-repaired lambs (B). AM-repaired lambs show an inability of the skin to heal over the lesion site.

skin only-repaired and unrepaired lambs may simply represent the benefit of increased protection from further intrauterine trauma secondary to use of a thicker patch for repair.

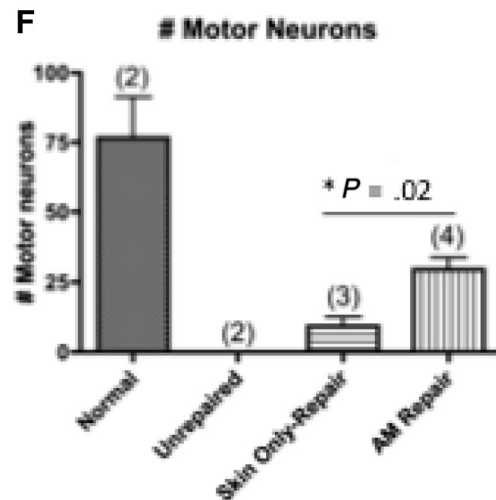
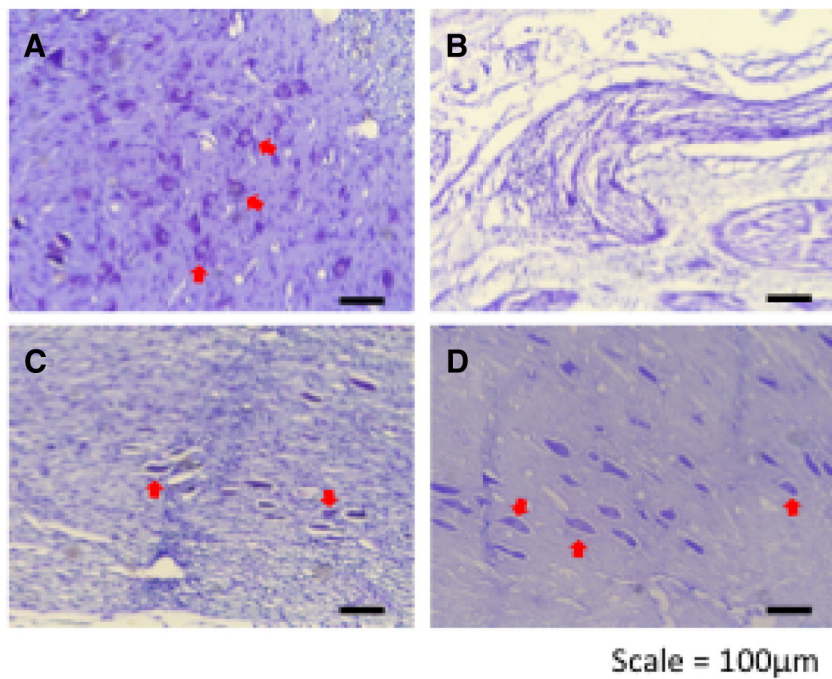
In addition to the role of the patch as a physical barrier, the AM may provide anti-inflammatory, paracrine, or regenerative

effects that mitigate loss of exposed tissue based on existing literature regarding the inherent immunoprotective qualities that the AM possesses [10,12]. Amniotic cells have been shown to secrete factors inhibitory to the innate and adaptive immune systems, to inhibit endothelial cell proliferation and angiogenesis,



**Fig. 2.** Cross-sectional images of the spinal cord at lumbar levels of normal, unrepaired, skin only-repaired, and AM-repaired lambs. Samples were stained with hematoxylin and eosin, Masson's trichrome, and cresyl violet. Unrepaired lambs show almost a complete loss of spinal cord tissue. Skin only-repaired lambs show some spinal cord tissue preservation with predominate thinning of the spinal cord as well as skin tethering to the cord (A). Quantification of the spinal cord tissue area present for each repair treatment was calculated using ImageJ; AM-repaired lambs show a significant increase in the amount of spinal cord tissue preserved. Numbers in parentheses are the number of lambs per treatment group (B).





**Fig. 3.** 10× images of cresyl violet stained lumbar spinal cord in normal (A), unrepaired (B), skin only-repaired (C), and AM-repaired (D) lambs. A selected number of motor neurons are indicated in each sample with red arrows. Unrepaired lambs are completely devoid of motor neurons. AM-repaired lambs show a significant increase in the number of preserved motor neurons compared to the skin only-repaired lambs. Numbers in parentheses are the number of lambs per treatment group (E).

and to secrete enzymes expressed by macrophages and neutrophils [16–18]. Furthermore, the literature supports the regenerative potential of amnion via stem cell effects. Over recent years, the placenta, amnion, and amniotic fluid have become increasingly investigated sources of stem cells. These gestational tissues are an attractive source for regenerative medicine as they are autologous, readily available, and can be harvested with acceptable risks to the mother and fetus. Mesenchymal stem cells (MSCs) have been harvested from the amnion, chorion, and amniotic fluid and shown to have similar properties to those MSCs derived from bone marrow [19–21]. Furthermore, another multipotent cell population, human amnion epithelial cells (hAECs), has been isolated from amnion and shown to have anti-inflammatory effects [21,22]. These immunoprotective and regenerative functions may play a protective role for developing spinal cord tissue and contribute to the preservation of neural tissue and motor neurons.

AM has also been shown to promote epithelialization. A prospective study using amnion in the treatment of venous ulcers demonstrated improved epithelialization and decreased fibrosis [6]. Another study found a decrease in fibrosis secondary to inhibition of TGF- $\beta$  with amnion [23]. In these models, it is thought that amnion promotes wound healing via epithelialization while simultaneously reducing scarring; this proposed activity could account for the decreased quantity of spinal cord adhesions seen in lambs treated with AM. This finding suggests a possible role in the reduction of tethered cord syndrome, a common complication of MMC repair that produces neurologic symptoms and necessitates further surgery.

Although the reduction of spinal cord adhesions in lambs treated with AM is encouraging, the concomitant wound-healing abnormalities warrant caution with regard to the potential use of AM in human MMC repair. Several studies have demonstrated the positive wound-healing effects of amnion for chronic wounds such as lower extremity

ulcers, full-thickness wounds, and necrotizing infections [6–10]; however, the opposite was seen in our studies, as surgical wounds failed to heal in lambs repaired with AM. From our two decades of prior fetal sheep experiments, we know that a fetal lamb will heal almost any wound, and it has been suggested that this is secondary to regenerative properties of the fetus [24]. It is possible that the inhibition of scarring in the fetal environment may prevent tissue remodeling and thus, interfere with wound healing in the fetus. The anti-inflammatory properties of AM may be so significant as to prevent adequate healing of the overlying dura and skin. While the open wound can be easily treated postnatally, the consequences of such wound-healing deficiencies in humans could result in failure to achieve a watertight repair. This would allow the CSF to continue to leak, and thus negate the beneficial effects of *in utero* repair on the Chiari malformation [25]. A future study could investigate the use of engineered gelatin sponges in conjunction with an AM patch as a means to promote tissue coverage as demonstrated in a rodent model of MMC by Watanabe, et al [26].

The current standard of care for prenatal repair of MMC is a skin only-repair as described in the MOMS trial [2]. The anecdotal success of one pretrial patient repaired with a fetal membrane patch followed by skin closure led to the present study. In this pretrial patient, the defect was too large to close with skin; therefore, an AM patch was harvested at the time of surgical repair and placed chorion side down over the exposed neural placode. For this reason, the same method was chosen for the present study. Areas of further investigation include reversing the directionality of the patch.

#### 4. Conclusion

This study details the first reported use of an AM patch during prenatal repair in a fetal model of MMC. These results suggest a potential role for AM as an improvement to skin closure. Lambs treated with AM during fetal MMC repair showed an exciting and significant increase in preserved spinal cord tissue and motor neurons as well as a decrease in adhesions to the spinal cord. These benefits, be they from the mechanical protection, anti-inflammatory properties, or regenerative functions of AM, are worthy of further investigation. Despite the potential benefits of using AM in future fetal MMC repair, the associated wound-healing deficits are not insignificant and should not be overlooked. While the neural tissue area and quantity of motor neurons present correlate with functional deficits in other animal models, we were unable to assess differences in motor function in the present study [14]. As improvements in functional recovery must also be assessed, future studies will compare the distal neurologic function of unrepaired, skin only-repaired, and AM-repaired lambs using an adaptation of the Basso-Beattie-Bresnahan (BBB) locomotor rating scale for spinal cord injury [27].

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#### Discussion

*Discussant: Dr. Tippi Mackenzie (San Francisco, CA):* You convinced us that the amniotic membrane has anti-inflammatory properties. Do you think that some of the effects that you saw could also be secondary to neural stem cells or other progenitors in the amniotic membrane that are contributing to this?

*Response: Dr. Erin Brown:* I think this is certainly an area for further investigation for us. From our preliminary studies we don't think that the amnion itself is a source of stem cells. There are numerous factors based on the literature that could contribute to these effects that we are seeing. Part of it could just be extra mechanical layer of protection over the cord and it is also likely that there are inherent paracrine or regenerative factors that are inherent to the amnion itself.

*Discussant: Omar Oda (Toronto, ON):* My question is, you artificially created the defects for this. Was it possible just to create it more naturally by, let's say, folate deficiency in these animals? Would it make any difference if you would create it more naturally than just artificially by surgery itself?

*Response: Dr. Erin Brown:* I'm sorry, could you repeat the question?

*Omar Oda:* You artificially created the defect to create meningocele, okay? My question is, if you could use the folate deficiency to create more natural defects and would that be different from creating it in a surgical way?

*Response: Dr. Erin Brown:* Absolutely. That is the inherent flaw of the sheep model, that it is a surgically created defect. In some other

animal species there are more developmental models such as rats and retinoic acid – we have heard about that. That is more beneficial but we have been unable to create a sort of developmental model in the sheep.

*Discussant: Dr. Shaun Kunisaki (Ann Arbor, MI):* Very nice study. I have two quick questions. One is, did you look more specifically at the degree of fibrosis and scarring? I know you showed some of the trichrome stains but were you able to sort of look closer at that since presumably that's the main effect of the membrane? My second question is were you able to gather any functional data at all from the survivors in terms of whether there was actually a difference?

*Response: Dr Erin Brown:* For your first question, yes, we do have some data on the amount of fibrosis for both the unrepaired and the skin repaired lambs. There was a significant amount of fibrosis and

scarring seen at the level of the defect and there was a significant amount less in the patch repaired lambs.

In terms of the motor function, we were hoping to gather some of that data but unfortunately we ended up with an n of 1 in each group, so while we subjectively think that the animal repaired with the patch had better motor function we can't really draw anything from the small numbers.

*Discussant: Dr. Ann Kosloske (Sanibel, FL):* Why don't you put the amnion right over the cord and then put a skin graft on top?

*Response: Dr. Erin Brown:* I worry with a skin graft on top of the amnion that we'd have similar results to what we saw in that the skin graft might have problems healing as well as just our simple skin closure over it. We certainly are thinking about other layers to put over this amnion patch to see if we would have problems with wound healing in that as well.