SKILL AND TALENT

Bladder changes after several coverage modalities in the surgically induced model of myelomeningocele in lambs

L. Burgos a,*, J.L. Encinas b, M.Á. García-Cabezas c, J.L. Peiró d, M. López-Santamaría b, E. Jaureguizar a

a Departamento de Urología Pediátrica, Hospital Universitario La Paz, Madrid, Spain
b Departamento de Cirugía Pediátrica, Hospital Universitario La Paz, Madrid, Spain
c Departamento de Anatomía Patológica, Hospital Universitario La Paz, Madrid, Spain
d Cirugía Pediátrica, Unidad de Cirugía Fetal y Neonatal, Hospital Vall d’Hebrón, Barcelona, Spain

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Abstract

Objective: To assess the presence of early bladder abnormalities in a prenatally corrected and uncorrected animal model of Myelomeningocele (MMC).

Method: A MMC-like lesion was surgically created in 18 fetal lambs between the 60th and the 80th day of gestation. Eight of them did not undergo fetal repair (group A), three were repaired with an open two-layer closure (group B), three using BioGlue® (group C) and four fetoscopically (group D). At term, bladders were examined macroscopically and histopathological changes were assessed using H–E and Masson Trichrome.

Results: Five animals in group A (5/8, 62%), two in group B (2/3, 66%), one in group C (1/3, 33%) and one in group D (1/4, 25%) survived. Macroscopically bladders in group A were severely dilated and showed thinner walls. Microscopically they showed a thin layer of collagenous tissue (Blue layer). BL lying immediately subjacent to the urothelium. The muscular layers were thinner. Non compliant pattern with thick wall and low capacity was also found in the non corrected model. Group B and the control showed preservation of muscular layers and absence of BL. Groups C and D presented BL but also preservation of muscular layers.

Conclusion: Bladder changes in a surgically induced model of MMC can be described using histopathological data. Both extremes of bladder changes can be observed in the model. These changes were completely prevented with open fetal surgery and partially with other coverage modalities.

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* Corresponding author.
E-mail address: lauraburgos33@hotmail.com (L. Burgos).

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PALABRAS CLAVE
Mielomeningocele; Fetal; Vejiga; Histopatología; Oveja; Cobertura; Capa azul

Cambios en la vejiga después de varias modalidades de cobertura en el modelo de mielomeningocele inducido quirúrgicamente en corderos

Resumen
Objetivo: Determinar las anomalías vesicales precoces en un modelo animal de mielomeningocele (MMC) con y sin corrección quirúrgica intraútero.
Método: Creamos una lesión similar al MMC en 18 fetos de cordero entre los días 60 y 80 de gestación. Ocho de ellos no se repararon prenatalmente (grupo A), 3 se intervinieron mediante cierre abierto en 2 planos (grupo B), 3 se cerraron con pegamento biológico (grupo C) y 4 por fetoscopia (grupo D). Al final de la gestación las vejigas se estudiaron macroscópicamente y histológicamente usando tinción de hematoxilina-eosina y tricrómico de Masson.
Resultados: Cinco animales del grupo A (5/8; 62%), 2 en el grupo B (2/3; 66%), uno en el grupo C (1/3; 33%) y uno en el grupo D (1/4; 25%) sobrevivieron. Macroscópicamente las vejigas del grupo A estaban muy dilatadas y sus paredes eran muy finas. Macroscópicamente mostraban una delgada capa de colágeno (capa azul [CA]) inmediatamente por debajo del urotelio; las capas musculares estaban muy adelgazadas. En el grupo no corregido también encontramos vejigas de baja acomodación, con paredes engrosadas y capacidad disminuida. El grupo B y el control mostraban preservación de las capas musculares y ausencia de CA. Los grupos C y D presentaban CA y preservación de las capas musculares.
Conclusión: Los cambios vesicales en el modelo quirúrgico de MMC en corderos pueden describirse mediante datos histopatológicos. Ambos extremos del espectro pueden darse en dicho modelo. Estos cambios pueden prevenirse por completo mediante cirugía fetal abierta y parcialmente a través de otras formas de cobertura.
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Introduction

Mielomeningocele (MMC) is a defect in the neural tube formation that represents the most frequent form of myelodisplasia with an incidence of 1/2000 live births. The lesion is characterized by the protrusion of meninges and medulla through the vertebral defect, leading to multiple disabilities at different levels: paraplegia, bladder and intestinal dysfunction, and Arnold-Chiari malformation.

Therefore, MMC is the most common cause of neurogenic bladder dysfunction in children (NBD). As it is known, NBD is accompanied by a number of well-described clinical and physiopathological patterns: loss of voluntary micturition control, detrusor overactivity, bladder/spincter dyssynergia, increased bladder pressure, and hypertrophy. The range of bladder changes ranges from an atomic poorly emptying bladder with miogenic failure to a non-compliant bladder, resulting in urinary incontinence and making necessary multiple surgical procedures, clean intermittent catheterization (CIC), and a close follow-up to achieve continence and avoid renal function deterioration.

It is postulated that MMC causes damage to the neural placode for two reasons (two-hit hypothesis): the original neural tube defect and the further exposure to the intrauterine environment and trauma. Several clinical and experimental studies have suggested that fetal surgical intervention to repair the defect could ameliorate this secondary lesion. Like the group of Philadelphia, which used a model of retinoic acid (RA)-induced MMC in fetal rats to assess functional and structural characteristics of the detrusor muscle in this NBD model. The resulting pathology has morphological and clinical similarities with human MMC, since the original neural tube defect and the posterior exposure are both present. They conclude that peripheral neural supply deteriorates throughout gestation and that, despite normal structural development, the functional status of the detrusor smooth muscle has already been altered in this model.3

On the other hand, the California group reported in 2006 an experimental work with adult female rats that underwent bilateral L5–S2 ventral root avulsion injury, followed, in some of them, by an acute implantation of the avulsed L6 and S1 roots into the conus medullaris. They concluded that implantation of avulsed roots promotes reinnervation of the urinary tract and return of micturition reflexes.4

Clinical studies of urodynamic outcome in patients with prenatally treated MMC have been reported by different groups and all of them agreed that the results were similar to those with conventional postnatal treatment.1,5-7

Histopathological bladder studies that could only be achieved with an experimental animal model could add some information to this clinical data. So, even though it is not a new concept and several groups had worked with different models, it remains a challenge.

The surgically induced model of MMC in sheep has strong resemblances with the human disease and the prevention of urinary incontinence after open fetal surgery has been described in this model; nevertheless, no histopathological bladder description has been reported previously.5,9

Our goal was to examine if this surgically induced model of MMC in sheep produces bladder changes similar to those found in human MMC. Finally, potential prevention of these changes using different prenatal treatments was also tested.
Material and methods

Animal housing

All our work was performed with approval of the Animal Care Committee of our Hospital in Madrid and according to European Council Directives (C86/609/EEC and 2003/65/EC) and Spanish guidelines (Boletín Oficial del Estado-BOE-of March 18, 1988). The experimental protocols were approved by the ethical committee at La Paz University Hospital.

All animals were obtained from a single breeder. Pregnant ewes were delivered one week before the intervention and housed in an appropriate establishment with free access to food and water except for the last 24 h before the intervention. An ultrasound (Toshiba® CK 700 S) study was performed prior to anesthetic proceedings to confirm pregnancy, determine fetal number, and position and estimate gestational age. We used two ultrasound parameters to establish gestational age: thoracic diameter and biparietal diameter.

Anesthetic procedures

Anesthetic induction employed midazolam 0.2–0.5 mg/kg i.m. and buprenorphine 0.01–0.02 mg/kg i.m. Fleece was sheared immediately before entering the operating room. Each ewe was positioned supine, intubated, anesthetized with propofol (10 mg/kg), and maintained with isoflurane 2–2.5% in oxygen 2 L/min. Placental transfer of the anesthetic gas provided fetal anesthesia. A single dose of the antibiotic prophylaxis (amoxicillin 15 mg/kg i.v.) was administered.

Surgical procedures

An MMC-like lesion was surgically created in 18 fetal lambs between day 60 and 80 of gestation as previously described8-10 (Fig. 1a). The 18 fetuses were distributed in 4 groups. 8 lambs (8/18; 44%) did not undergo fetal repair (group A), 3 animals (3/18; 16%) were repaired with open closure, 3 lambs (3/18; 16%) with open biogluce coverage (group C), and 4 (4/18; 22%) with percutaneous fetoscopic coverage using a patch (group D) (Table 1).

After 30 days, defects in group B were repaired with an open two-layer closure of the placode/duro complex and skin using 6.0 and 5.0 PDS (Fig. 1b). The defect in group C was covered by direct application of biogluce (Cosseal, Baxter®) (Fig. 1c). In group D, if the volume of amniotic fluid volume was too scant to permit a safe placement of the first trocar, a 20-gauge needle was introduced under ultrasound guidance. Visibility was improved by low pressure (5 mm Hg) Helium inflation of the amniotic cavity. Coverage was approached using a Duragen patch applied with a running suture (Fig. 1d). Two bladders from normal unoperated animals served as controls.

Delivery and postnatal care

Fetuses were allowed to go to term (day 140) and were delivered vaginally. Lambs were housed in warm places with their mothers and all of them were sacrificed within 24 h and then perfused with paraformaldehyde 7%.

Histopathological procedures

Kidneys, ureters and bladders were extracted “en-block”, photographed and studied for signs of hydronephrosis, ureteral distension, and changes in the external appearance of bladder wall. Kidneys were cut and opened like a book and bladders and ureters sectioned by 1-cm deep transversal cuts. To make comparisons between bladders, samples were taken from posterior detrusor muscle. Photographs were taken from transversal sections (Nikon D40X 10 mp camera). Blocks were formalin-fixed and paraffin-embedded and 6 μm sections were obtained and stained with Hematoxilin–Eosin and Masson Trichrome. Sections were examined under a Leica DM LS2. Photomicrographs were taken with a digital camera Leica EC3 and the software provided by the manufacturer.

Results

Nine fetuses died during pregnancy (9/18, 50%) with one instance of maternal death (1/18, 5%) due to pulmonary embolia. Five animals in group A (5/8, 62%), two in group B (2/3, 66%), one in group C (1/3, 33%) and one in group D (1/4, 25%) (n.s.) reached term and were born between day 140 and 145 of pregnancy (Table 1). Lambs in group A (non-corrected animals) were incontinent for urine and showed flaccid paraplegia, muscular hypotrophy, and absent reflexes, but since they were sacrificed in the first 24 h of life, little more can be said about the clinical aspects. Our high mortality rate was due to preterm delivery probably secondary to choioamnionitis, but anyhow it is similar to mortality published by other groups.9,11 Of course, results were analyzed bearing in mind the low number of cases and more population would be necessary to achieve conclusions.

Macroscopic

Animals without prenatal coverage (group A) showed the two opposite patterns presented in human disease: dilated and thin bladders (Fig. 2a) or contracted bladders with high

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of lambs</th>
<th>Closure technique</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>n = 8</td>
<td>No repair</td>
<td>5/8 (62%)</td>
</tr>
<tr>
<td>Group B</td>
<td>n = 3</td>
<td>Open fetal closure</td>
<td>2/3 (66%)</td>
</tr>
<tr>
<td>Group C</td>
<td>n = 3</td>
<td>Open biogluce coverage</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td>Group D</td>
<td>n = 4</td>
<td>Percutaneous fetoscopic patch coverage</td>
<td>1/4 (25%)</td>
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</tbody>
</table>
thickening (Fig. 2b), both of them resembling the non-treated neurogenic-bladder dysfunction (NBD) patterns found in human MMC. Macroscopic bladder appearance from any other group was normal.

Microscopic description

As in the macroscopic description, animals without treatment (group A) presented the 2 opposite patterns. Four of them presented thin muscular layers with great separation and fibrosis between the smooth muscle bundles. The urothelial layer only had one or two cells (Fig. 2c). On the other hand, the opposite pattern was found in one case of non-corrected MMC, with marked thickened muscular layer and 6 or 7 cells in the urothelium. No intramuscular fibrosis was recognized in this case (Fig. 2d).

Non-corrected animals presented dense fibrosis in the subepithelial connective tissue described as a blue submucosal layer (BL) (Fig. 2c and d). It was not observed in any of the normal animals or in those corrected with open fetal surgery or patch coverage.

In group B, those corrected with an open two-layer closure of the placode–dura complex, the most relevant finding was the absence of gross lesions and their similitude to normal bladders. Urothelium and muscular layer were preserved and there was not BL.

Animals treated with biogel (group C) and those closed by fetoscopy using a patch (group D), showed mild changes with moderate separation between muscular bundles, a median number of urothelial cells, and a blue fibrous layer (Table 2).

Ganglia (Fig. 2e) and nerves (Fig. 2f) were present and normally developed in every animal.

No malformations in ureters or kidneys were produced in any group.

Discussion

The MMC sheep model does not present the cord damage present in human MMC that is supposedly intrinsic to the disease. Nevertheless, it does reproduce the clinical symptoms of paraplegia and urinary incontinence and some of the main histological findings in brain, medulla, and rectum of human MMC. As we already published in 2008, some of the central

Table 2 Micro and macroscopic appearance of each treatment group.

<table>
<thead>
<tr>
<th>Macroscopic results</th>
<th>Microscopic results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (no coverage)</td>
<td>Dilated thin bladders (n = 4)</td>
</tr>
<tr>
<td></td>
<td>-1–2 urothelial cells</td>
</tr>
<tr>
<td></td>
<td>-Thin muscular layer</td>
</tr>
<tr>
<td></td>
<td>-Great separation and fibrosis</td>
</tr>
<tr>
<td></td>
<td>-Blue layer</td>
</tr>
<tr>
<td>Group B (open closure)</td>
<td>Thick non compliant bladder (n = 1)</td>
</tr>
<tr>
<td></td>
<td>-6–7 urothelial cells</td>
</tr>
<tr>
<td></td>
<td>-Thick muscular layer</td>
</tr>
<tr>
<td></td>
<td>-No intramuscular fibrosis</td>
</tr>
<tr>
<td></td>
<td>-Blue layer</td>
</tr>
<tr>
<td>Group C (biogel coverage)</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>-Urothelium and muscular layer preserved</td>
</tr>
<tr>
<td></td>
<td>-No blue layer</td>
</tr>
<tr>
<td>Group D (fetoscopic path coverage)</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>-4 urothelial cells</td>
</tr>
<tr>
<td></td>
<td>-Moderate cells separation</td>
</tr>
<tr>
<td></td>
<td>-Blue layer</td>
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</tbody>
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Figure 2 Pathological aspects. (A) and (B) Macroscopic view of non-corrected bladders showing opposite patterns: (A) distended and thin and (B) extremely thickened. (C) and (D) Histologic examination of the bladder wall in non-corrected animals. (C) Thinning and distension with separation between the muscle bundles and presence of fibrous tissue. Urothelium formed by one or two cells. Presence of BL (black arrow). (D) Marked thickening of the bladder wall. Urothelium formed by 6 or 7 cells. Presence of BL (white arrow). (E) and (F) Histologic examination of the nerves and ganglia in non-corrected animals. (E) Normal ganglia (black arrow). (F) Presence of normal neural tissue (black arrow) suggesting the functional impairment as the origin of the lesions observed in the bladder.

nervous system abnormalities associated to MMC in human patients were also found in the uncorrected fetal lamb model of MMC, in terms of ependymal denudation, hydrocephalus, and Arnold-Chiari malformation. So, although we should consider the intrinsic limitations of this particular model, a pathological study could assess the effects on the bladder that would only be related to the environmental exposure of the cord and to the different coverage modalities used.

In our study, animals without coverage (group A) showed a highly dilated bladder with paucity of muscular layers and the presence of mild fibrous tissue between the layers. The
histological structure was preserved, so these mild changes suggest that the bladder was developed in conditions of deficient emptying during gestation. In fact, one of the few purely pathological studies that consider fetal or neonatal human bladders with MMC reports some alterations similar to those found in the model used here. Shapiro et al. described how in four out of five stillborn MMC fetuses, the muscle bundles were fewer and significantly diminished in size compared to normal fetuses, which is similar to our findings in most of the animals of the non-treated group.\(^{11}\)

On the contrary, in our series, we found the opposite changes in one case: there was a markedly thickened wall with extremely coarse muscle layer that could resemble the non-compliant trabeculated bladder also found in human MMC. Although this is just one specimen, it could be argued that a lesion in the medullary pathways may lead the bladder to one or the other extreme of the spectrum depending on the lesion level. Thus, as Shapiro suggests, perhaps intact innervation of the bladder may be a prerequisite for the normal program of morphogenesis to proceed with the development and organization of bladder smooth muscle.\(^{11}\)

The presence of the particular finding of a BL of fibrous tissue under the urothelium in every animal except for controls or those treated with open fetal surgery, could suggest some kind of urothelial damage. We hypothesized that, in the context of inadequate emptying, this fibrotic band could be produced as a stress response, in the same way that bladder fibrous tissue has been described in the presence of obstructive malformations in the sheep model.\(^{12,13}\)

However, morphometric analysis of the bladder wall thickness was not performed because this variable is dependent on bladder distension during fixation that could not be standardized. Since muscular cells distribution is not homogeneous, to determine the exact number of cells and collagen fibers found, it would be necessary to assess a referential surface and, therefore, to perform a complete seriation of the bladder wall. Our limited analysis did not allow us to do so.

However, none of the described changes were found in the normal controls or in those bladders corrected using open fetal surgery and they are similar to neonatal findings of human disease.

The most relevant finding in the bladders from animals with open fetal surgery coverage (group B) was the absence of gross lesions and their similitude to normal bladders. This suggests that this particular modality of coverage (similar to the technique used in humans inside the MOMS study) (2) is useful in preventing bladder malformations in the surgically induced MMC model.

Coverage with bioglue (group C) is a simpler technique that has potential application in fetoscopic treatment. Although lumbar lesion appearance was very similar to the non-covered animals, we have found that a dense layer of fibrous tissue is observed at term covering the defect. Pathological findings in this group were variable within the only specimen available (Table 2) presenting just a part of the benefits found with open surgery.

The percutaneous fetoscopic coverage using a patch (group D) has been reported elsewhere\(^{14}\) and our only relevant conclusion could be the extreme difficulty related to the procedure. Bladder findings from the only survivor from this group were very similar to observations in controls or open fetal surgery group.

Finally, we did not find any renal or ureteral malformations in the model. As it is well known, those malformations are frequently described in MMC but usually in later stages of the disease.\(^{11}\)

There are not too many experimental animal studies focused on this particular problem. The Philadelphia group, using retinoic acid induced MMC in a rat model, demonstrated sphincter dysynergia, but no morphological changes.\(^{3}\) Some authors have described urinary bladder malformations in sheep using a model of urinary obstruction. Interestingly, most of the histopathological observations in the non-treated animals of our study in terms of bladder muscular thinning and fibrosis, showed a strong resemblance with the results in the partial or functional obstruction group.\(^{12,13}\)

From the clinical point of view, little information is available describing the clinical evolution of prenatally treated children with MMC focused on their postnatal urological symptoms. Urologic outcome in infants who underwent prenatal closure of MMC have only showed an absence of remarkable clinical improvement in several series.\(^{5,5,11,12}\)

Conclusion

Bladder changes in a surgically induced model of MMC can be described using histopathological data.

Our results suggest a strong resemblance between the surgically induced model of medullary exposure and the human disease with both extremes of bladder changes being described in this model. Despite the small number of cases, open fetal surgery seems to avoid most of histopathological lesions presented in MMC bladder at birth and other coverage modalities could prevent them partially.

A functional approach to bladder disorders using this model could be developed in the future based on the study of differences in bladder volumes or in the proportions of collagen and elastine.

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Conflict of interest

The authors declare that they have no conflict of interest.

References