Fetal therapy for spina bifida in a sheep model
using tissue engineering

Foetale therapie bij spina bifida in een schapenmodel met toepassing van weefseltechnologie

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van de Medische Wetenschappen

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Alexander Jan Eggink
geboren op 8 september 1968
te Rotterdam

Uitgeverij Valkhof Pers, Nijmegen

PROMOTOREN
Prof. dr. W.F.J. Feitz
Prof. dr. P.P. van den Berg, Universitair Medisch Centrum Groningen
Prof. dr. F.K. Lotgering

COPROMOTOREN
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Dr. P.J. Geutjes

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Prof. dr. J.A. Deprest, Universitair Ziekenhuis Leuven, België
Dr. M.A.A.P. Willemsen

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Omslagontwerp & lay out: Brigitte Slangen, Nijmegen
Opmaak binnenwerk & illustratiekatern: Peter Tychon, Wijchen

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CHAPTER I

General introduction, aims and outline of the thesis

Introduction

Spina bifida (SB) means 'split spine' and is one of the most prevalent major birth malformations with a worldwide incidence of approximately one in 2,000 live births [1-3]. It can result in lifelong impairment of the lower extremities, bowel and bladder dysfunction, sexual dysfunction and mental impairment. Although spina bifida is a nonlethal condition, it can be a devastating disorder because of the long-term severe morbidity [4, 5]. Spina bifida is one of the most common birth defects involving the central nervous system. In addition to spina bifida; anencephaly, encephalocele, and, although rare, craniorachischisis and iniencephaly are within the clinical spectrum of neural tube defects (fig. 1).

Neural tube defects (NTDs) result from abnormal development of the brain or spine. In spina bifida aperta; spinal cord, nerves and meninges protrude through a defect of the vertebral arch, muscle and skin, most commonly in the lumbar and sacral regions of the spine (fig. 2). Such in contrast with spina bifida occulta in which only a defect of one vertebral body is present, covered by skin, and generally without functional anomalies. A myelomeningocele (MMC) is the most frequent form of spina bifida, characterized by the extrusion of the spinal cord and/or nerves through a bony defect of the spine into a sac filled with cerebrospinal fluid (CSF).

Based on a prevalence of 10 per 10, 000 births, each year more than 4, 500 pregnancies in the European Union are affected by NTDs (anencephaly, spina bifida or encephalocele) [6]. Worldwide, each year approximately 300,000 newborns are born with a NTD [7]. In The Netherlands, the prevalence of isolated spina bifida was 4.1 per 10, 000 births in the period 2000-2009 (Eurocat Update: Actual numbers congenital anomalies in Northern Netherlands 1981-2009). Spina bifida accounted for 57% of all NTDs followed by anencephaly (33%) and encephalocele (10%). In 45% of the cases with spina bifida registered within the Dutch registration of Eurocat, parents decided to terminate pregnancy after prenatal diagnosis. This is consistent

with numbers from the USA however, in some areas of the Netherlands, this figure is considerably higher [8]. Since the introduction of the routine 20-weeks ultrasonographic screening in The Netherlands, the number of prenatally detected spina bifida before 24 weeks' gestation increased and consequently also did the number of elective terminations of pregnancy because of spina bifida before 24 weeks' gestation. Still, 61% of all newborns with a spina bifida were life born in the period 1999-2008 (Eurocat Update: Actual numbers congenital anomalies in Northern Netherlands 2010). Twelve percent ends in miscarriage or stillbirth.

Etiology

NTDs are complex multifactorial defects. Interaction with external agents, like environmental factors, may trigger or suppress a genetic predisposition [9]. Geography, race and ethnicity, nutrition, medication, maternal illness, socioeconomic status, family history and previous affected pregnancy are important epidemiologic determinants of NTDs [7, 10]. Together with long-term trends over time, they suggest the importance of environmental and genetic contributions. However, the cause is not known in most cases [11]. North China shows a high prevalence of NTDs and within the USA a higher prevalence in Hispanic/non-Hispanic whites is found comparing to African-American and Asian women [7, 12]. Prevalence is also higher with lower socioeconomic status [7]. Important established risk factors with an increased relative risk (RR) for spina bifida includes: history of previous affected pregnancy with same partner (RR 30), valproic acid and carbamazepine use (RR 10-20), pregestational maternal diabetes (RR 2-10) and inadequate maternal intake of folic acid (RR 2-8) [12]. Also, the risk of having a child with spina bifida is increased in obese women (RR 1.5-3.5), probably attributable to hyperinsulinaemia [12]. In the literature, girls tend to be more affected than boys however, this could not be confirmed in the Dutch Eurocat registration [1, 12].

Both multiple congenital anomalies and genetic syndromes, especially trisomy 13 (Patau syndrome) and 18 (Edwards syndrome) are associated with spina bifida. However, syndromal NTDs represent only a minor part of all NTD cases. In 71% of all cases no other syndromes or anomalies are found (Eurocat Update: Actual numbers congenital anomalies in Northern Netherlands 2010) [9]. Nutritional factors are associated with NTD formation. Maternal deficiencies of certain micronutrients or vitamins, especially folate,

play an important role in the pathogenesis of NTDs [1, 10]. Studies showed that women, pregnant of a child with a neural tube defect, have a lower serum level of vitamin B12 and inadequate intake of folic acid, before and during early pregnancy [7, 12]. Folates can be provided by both natural and fortified foods, as well as by supplementation. Improving folate status by folic acid supplementation reduces the risk as well as the recurrence risk of having a child with a neural tube defect [7, 9, 10, 13]. Several studies demonstrated that periconceptional supplementation of folic acid may prevent up to 70% of all NTDs [1, 6, 9]. These results led to the introduction of a periconceptional folic acid supplementation policy, formulated by the government in The Netherlands in 1993. Also a deficiency of vitamin B12 is associated with neural tube defects and disorders in the metabolism of folate or B12 can result in elevated homocysteine levels [10]. Studies showed that both the fasting homocysteine level and the homocysteine levels before and after the intake of methionine (methionine-load test) are elevated in women, pregnant of a child with spina bifida [10]. Despite the knowledge that improving folate status prevents more than two thirds of all neural tube defects, the prevalence of neural tube defects has not declined significantly in Europe between 1980 and 2001 [6]. The reduction in live birth NTD prevalence relies more on prenatal screening and termination than on primary prevention. Also in the Dutch Eurocat registration there was no substantial decline in prevalence of spina bifida [14]. Intake of folic acid is important during the periconceptional period, especially the first 28 days and in unplanned pregnancies, most women will realize they are pregnant after this period [5]. Also, 50% of all women did not routinely take periconceptional folic acid even one year after the campaign of the government in The Netherlands [15]. Despite of the fact that the goal of preventing NTDs with periconceptional folic acid supplementation has not been reached, public health interventions to encourage and increase folic acid use should be continued.

Embryology

In the embryonic period, spinal cord and brain are developed from the neural tube. The sequence of events that form the neural tube is called neurulation. In the process of primary neurulation the cellular plate is formed. Folding of the neural plate by invagination leads to formation of the neural folds and neural groove. The groove gradually deepens as the neural folds become elevated, and ultimately the folds meet, fuse in the midline, and convert the

neural groove into the neural tube. The most caudal part of the spinal cord is formed by secondary neurulation. The process of development and closure of the neural tube is completed approximately 25th days post-conception. From the caudal part of the neural tube the spinal cord is formed. The brain develops from the cranial part. Vertebral arches, muscles and surrounding tissues are formed by migration of mesodermal cells around the neural tube and form a protective barrier [5]. Neural tube defects are disorders of primary neurulation [16]. Failure of closure of the cranial neural folds leads to anencephaly. An abnormal primary neurulation with failure of fusion in the caudal regions of the neural tube leads to spina bifida. Bone and muscle are unable to grow over the open section of the neural tube, thus resulting in a defect through which the spinal cord, nerves and meninges protrude [1]. The neuro-epithelium develops into a plate instead of a tube and the neural plate develops at the level of the defect into a dysplastic and malformed myelum called neural plaque or placode.

Symptomatology

The severity of symptoms is correlated with the level of the defect. Interruption of the spinal cord at the site of the defect can cause lifelong paralysis of the legs, bowel and bladder dysfunction, sensibility disorders of the skin, sexual dysfunction and deformation of the lower extremities and back. Most children with spina bifida are not mentally retarded but their intelligence quotient can be reduced [8, 17]. Although spina bifida can be compatible with independent life, lifelong supportive care is often needed and only about 50% of the patients are able to live independently as adults, even with adapted accommodations [3-5, 17, 18]. In brief, approximately two-thirds of children with spina bifida may reasonably expect to ambulate the majority of the time [17]. Only 1% of children born with an open spina bifida are free of handicaps [1]. Sacral lesions caudal to the first sacral level have the best prognosis for independent walking. Almost all lesions cranial to the second lumbar level lead to wheelchair dependency and scoliosis [9]. In addition, lesions at the thoracal level have the worst prognosis. Through the years, sensorimotor deficits in the lower extremities can worsen because of deformities of the lower extremities, orthopedic problems of the back (kyphosis and scoliosis) and tethered cord syndrome (adherence of the distal spinal cord or filum to adjacent structures most commonly to the area of the original repair) [4, 19]. Also social problems, attributable to fecal and urinary incontinence and sex-

ual dysfunction become more prominent in adolescence [2]. The majority of children with spina bifida have a Chiari II malformation (CM) which is a combination of hindbrain herniation and hydrocephalus, found only in patients with spina bifida [4]. Some believe the CM is caused by the leakage of CSF through the neural tube defect which causes the downward displacement of the hindbrain and collapse of the primitive ventricular system because of the pressure gradient [20]. Hydrocephalus is caused by the elongation and obliteration of the fourth ventricle due to downward herniation of the cerebellum and brainstem [21, 22]. Others believe hydrocephalus is secondary to maldevelopment of the CSF pathway in the posterior fossa [23]. It is hypothesized that the volume of the posterior fossa in spina bifida patients is small. In the past, it was believed that hindbrain herniation was part of an overall cerebrospinal dysgenesis, but experimental and clinical evidence has shown that both hindbrain herniation and hydrocephalus are acquired early in fetal life and progress in severity before birth [24, 25]. The CM is the leading cause of death in patients with spina bifida [25, 26]. Seventy-five percent of deaths in neonates and children with spina bifida are attributable to hindbrain dysfunction [24]. Hydrocephalus is present in more than 85% of all patients and 80-90% of children with hydrocephalus will receive a CSF shunt to prevent additional damage to brain and brainstem [1, 9, 21, 24, 25]. The upper level of the spina bifida lesion appears to be a major determinant of the need for shunt placement [17]. Because of complications like mechanical problems or central nervous system infection, shunt revision is necessary in half of the patients [4, 24]. Complications and shunt revision can have a negative influence on intellectual development and hydrocephalus remains one of the main reasons for long-term morbidity and mortality [1, 4, 26, 27]. Before the introduction of neurosurgical treatment and shunt placement in the sixties, mortality was highly attributable to hydrocephalus, meningitis and perinatal problems. Despite the successes of postnatal neurosurgical repair and medical treatment of spina bifida, mortality still remains approximately 10%, rising to 35% in those children with symptoms of brainstem dysfunction secondary to the CM [1, 2, 4, 7, 9, 21, 28].

Diagnosis

Neural tube defects can be detected by ultrasound in the first and second trimester of pregnancy. Besides the typical U-shaped defect of the spinal cord of a fetus with an open spina bifida, the CM leads to the typical lemon shape of the fetal skull and the banana shape of the cerebellum and in most cases ventriculomegaly in the second trimester [29]. In some cases, microcephaly is also present [29]. Club feet are present in most cases. A recent study suggests that in open spina bifida caudal displacement of the brain is evident from the first trimester, resulting in compression of the fourth ventricle and loss of the normal intracranial translucency [30]. Since the increasing use of routine ultrasonography screening at 20 weeks' of gestation in The Netherlands, being part of routine prenatal care, prenatal detection of spina bifida before 24 weeks' gestation has increased [31]. Besides ultrasound, spina bifida can also be detected by elevated levels of alpha-fetoprotein in the amniotic fluid after amniocenteses. With the high detection rate of the ultrasound scan to diagnose spina bifida, biochemical assessment of the AF has been replaced by ultrasonography [30].

In the region of Nijmegen, The Netherlands, all women, prenatally diagnosed with a spina bifida of their child are referred to the tertiary centre of Prenatal Diagnosis and Therapy at the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. After ultrasound and accurate assessment, the parents are informed about the results and the prognosis by the pediatric neurologist and perinatologist in a multidisciplinary approach. Amniocentesis is offered in every case because of the elevated risk of additional chromosomal abnormalities. A social worker offers the parents emotional support. All referred patients are advised to give birth in a tertiary centre to guarantee optimal care of the newborn after birth and to prevent delay in further diagnostic evaluation and surgical closure of the defect.

Delivery

Whether delivery of an infant with a prenatally diagnosed spina bifida should be by elective cesarean section or by vaginal birth remains controversial. Some studies have suggested improved motor function with elective cesarean section prior to the onset of labor or rupture of membranes but others could demonstrate no positive effect on the neurological outcome [32-36].

Prospective randomized controlled trials, comparing routes of delivery have not been performed. Elective cesarean section is often offered to the parents to protect the spinal cord from injury because of suggested pressure on the neural tissue and to prevent possible rupture of the meningeal sac [3, 37]. However, there is no conclusive evidence that cesarean section improves the outcome in children with spina bifida relative to vaginal delivery. In case of breech presentation or large defects, it might be justified to deliver by elective cesarean section to reduce the risk of trauma [12, 35].

Treatment

The current standard of postnatal care is neurosurgical closure of the defect. Main goals for surgery are to untether the placode, eliminate the liquor leak, prevent infection, preserve neural functions by preventing further damage to the cord and to prevent secondary tethering of the spinal cord at the site of surgical repair [4]. Surgical intervention does not improve neurological function but prevents further deterioration. Once a child with spina bifida is delivered, primary closure of the defect is performed within 48-72 hours of birth [1, 4, 5]. Before intervention, an extensive neurological evaluation is performed to assess the level of the lesion and prognosis for motor ability, the presence of hydrocephalus, deformities of the leg and leg movement. Also an urological evaluation is performed. If necessary, a ventriculoperitoneal shunt is placed to drain excess CSF from the brain into the abdomen where it is absorbed.

Experimental therapy and the rationale for in utero repair

The question whether the neurological disorders associated with spina bifida are caused by either a primary disorder of neurulation during the embryonic development of the spinal cord or by secondary damage to a primarily normal spinal cord caused by the intrauterine environment or both, has been studied and described extensively in the literature [16, 38-43]. Heffez et al. [16] demonstrated in a rat model with a surgically created dysraphism that exposing the spinal cord to the amniotic fluid induced paralysis of the hindlimbs and that significant neurological deficits occur after only 48 hours of exposure. Meuli et al. [38] showed in his histological study of fetuses with spina bifida, born after termination of pregnancy between 19-23 weeks' gestation,

that there is good development of spinal cord tissue, nerve roots, and ganglia within the area of the defect and that the phenomenon that accounts for the loss of neurological function is most likely a traumatic or toxic destruction of neural tissue during further gestation. In a study of mice there were no signs of significant traumatic or degenerative changes within the exposed spinal cord in fetuses with spina bifida at early stages of gestation. Results at a later gestational age provided evidence for secondary degenerative changes which progress with ongoing gestation to partial or complete loss of all exposed neural structures by birth [44]. Leg movement has been found on sonograms of affected fetuses before 17 to 20 weeks, whereas later in gestation and in neonates, some degree of deformity and paralysis was present [45]. The lower limb movements noted early in gestation could be secondary to spinal arc reflexes. However, such movements could be of cerebral origin and their absence in later gestation may be attributable to neural tissue damage.

This knowledge led to the 'two-hit' hypothesis; failure of primary neurulation in the embryonic period leads to the development of myelodysplasia (first-hit) and due to the absence of skin and musculoskeletal coverage, the persistent exposure of the openly exposed neural tissue to the intrauterine environment can lead to secondary acquired neural tissue damage and, consequently, irreversible loss of neurological function (second-hit) [16, 46, 47]. It is believed that part of this acquired damage is caused by the amniotic fluid (AF) [9, 47]. Experiments suggest that late gestation AF might be toxic to the exposed neural tissue and cause chemical injury [39]. This toxicity may be explained by the increased concentration of urea, especially in the third trimester, the presence of gastrointestinal waste products, like digestive enzymes, and the lower osmotic pressure which results in an osmotic pressure gradient between AF and neural tissue [16, 48-54]. Destruction can also be caused by mechanical shearing and abrasive stresses on the surface of the delicate neural tissue, especially in the third trimester when there is increasingly less AF with a greater probability of contact injury of the exposed spinal cord to the amniotic wall and during delivery [55].

This 'two-hit' hypothesis is the rationale for in utero repair of a spina bifida implicating that the second hit can be prevented by intrauterine closure of the defect [46]. Prenatal coverage would stop the progressive neural tissue destruction and improve the neurological outcome at birth. Also, in utero coverage of the defect may stop the leakage of CSF and consequently have a positive effect on the associated cerebral anomalies like hindbrain hernia-

tion and hydrocephalus [26]. This theoretical basis for intrauterine repair was first proposed by Heffez et al. [16]. Later, Meuli et al. [40, 47, 56] showed in a sheep model that in utero repair of an experimental spina bifida stops the ongoing process of spinal cord destruction and preserves neurological function. Paek et al. [21] demonstrated that prenatal repair prevents or reverses development of hindbrain herniation in a fetal lamb model. Although the animal model has its limitations which need to be considered when interpreting results, it is widely used to study the effects of intrauterine repair of spina bifida and for evaluating new techniques [20-22, 40, 41, 47, 56-66].

The first cases of in utero coverage of spina bifida in humans were performed in 1994 with an endoscopic technique [48, 67]. At that moment, the technique proved unsatisfactory and was abandoned. In 1997, the first intrauterine repair of spina bifida by hysterotomy was performed [68, 69]. The results of these first studies suggested improvement in hindbrain herniation and a significant reduction in shunt-dependent hydrocephalus relative to infants treated postnatally [3, 9, 25, 68, 70-72]. The mechanism of reversal of hindbrain herniation remained undefined but shunt status affects cognitive functioning in later life and is therefore a surrogate marker for long-term sequelae. Improvements in sensorimotor function and urodynamic function were disappointing and similar in infants treated in utero compared to infants treated postnatally [12, 72, 73]. One of the potential risks of open fetal surgery is premature delivery. Mean gestational age at delivery after open fetal surgery is approximately 32-34 weeks' gestation [8, 59, 70, 71, 74]. Extreme premature delivery (< 30 weeks) was seen in 11-13% of the patients [70, 74]. Other complications described are; chorioamnionitis, rupture of membranes, separation of the amniotic and chorionic membrane, oligohydramnios, placental abruption, uterine rupture, dehiscence or a very thin uterine wall at the hysterotomy site at the time of delivery, pulmonary edema as side effect of tocolytics, neurological injury of the fetus, fetal demise, respiratory distress syndrome in the neonate, peroperative complications like bleeding and transfusions, and the need for cesarean section in subsequent pregnancies [69, 72, 74, 75]. There have been no maternal deaths in any fetal surgery series [8].

From 2003 through December 2010, intrauterine repair of spina bifida was almost only practiced in the three participating centers of the MOMS trial (Management Of Myelomeningocele Study) in the USA; the University of California, San Francisco, California, The Children's Hospital of Philadelphia,

Pennsylvania and Vanderbilt University Medical Center in Nashville, Tennessee [74,76,77]. This randomized controlled three-center trial compared the two approaches to the treatment of children with spina bifida; fetal surgery before 26 weeks of gestation and standard postnatal repair. The trial was stopped for efficacy of prenatal surgery after the recruitment of 183 of a planned 200 patients (91 fetal and 92 postnatal repairs). One primary objective was to determine if intrauterine repair at 19-25 weeks' of gestation improves outcome, as measured by fetal or neonatal death or the need for shunting by the age of 12 months, compared to postnatal repair [55,74]. Another primary outcome at 30 months was a composite of the child's mental development and motor function. Prenatal surgery was done at one of the three MOMS centers between the 19th and 25th week of pregnancy. Open fetal surgery was performed under maternal general and epidural anesthesia providing adequate fetal anesthesia and uterine relaxation [5,8]. After hysterotomy the fetus was positioned with the spina bifida lesion visible through the uterine incision. The spina bifida closure was similar to the standard postnatal closure. The cystic membrane of the spina bifida was carefully excised from the placode and the attachments of the meninges to the skin and soft tissues were detached. If possible, dura was closed over the spinal cord as a first layer. Paraspinal myofascial flaps and carefully mobilized surrounding skin were closed over the defect. Every attempt was made to close it primarily. If not possible, due to the size of the defect, an acellular human dermis graft patch was used to complete the closure [8,55]. Delivery by cesarean section around 37 weeks' gestation was mandatory to prevent uterine rupture in the stapled scar. This clinical trial demonstrated that prenatal surgery for spina bifida reduced the need for shunting (actual rates of shunt placement 40% and 82% in the prenatal-surgery group and postnatal-surgery group respectively) and improved the composite score for mental development and motor function at 30 months as compared with postnatal surgery, but was also associated with an increased risk of preterm delivery and uterine dehiscence at delivery [74]. In the prenatal surgery group, fetuses were born at an average gestational age of 34.1 weeks compared with 37.3 weeks in the postnatal surgery group.

Most of the complications of fetal therapy are associated with open fetal surgery and although the endoscopic coverage may reduce these maternal risks, technically this minimally invasive approach still remains a challenge [5]. A pilot study for the development of a percutaneous fetoscopic approach was done in Germany [78]. Percutaneous fetoscopic access could be estab-

lished with 3 ports with an external diameter of 5 mm. In order to overcome the disadvantages of an in-fluid operative approach, partial amniotic carbon dioxide insufflation was used to maintain clear visualization throughout the procedure. The spina bifida was covered with patch material and skin after dissection of the placode and resection of pathological tissues [78]. However, also with the endoscopic approach, preterm delivery has been a substantial problem, probably related to the membranous damage [79].

Tissue engineering

Especially with large defects, primarily, tension-free, in utero closure with skin is sometimes not possible and a graft or patch may be used. Also in early repair, fetal tissue lacks the integrity required for extensive dissection and suturing. In human studies and in animal experiments, a great variety of different materials have been used to cover the spina bifida lesion [20,41,59,61,65,80-83]. Most of the materials are not specifically designed for fetal surgery but have been widely used in other fields of medicine.

It is a challenge to develop a tissue-engineered construct, specifically tailored to the needs of surgical reconstruction of birth defects in the fetal period, particular when looking at the prerequisites of fetal surgery. Tissue engineering is a fast growing interdisciplinary field that applies the principles of engineering and life sciences to the development of biological substitutes that restore, maintain, or improve tissue function [84-86]. A scaffold is a three-dimensional matrix, sourced from natural or biosynthetic materials, that serves as an artificial extracellular matrix (ECM) for cellular migration from the surrounding tissue, adhesion, proliferation, and tissue regeneration in three dimensions. Scaffolds based on an ECM protein, like collagen, can be prepared with both a known architecture and defined composition [87,88]. Special demands are put upon scaffolds and their preparation when used for fetal treatment of spina bifida. The material should be pure, not toxic with a low antigenicity, biocompatible and biodegradable, have appropriate mechanical strength and a porous matrix structure to allow cell migration, enhance tissue ingrowth and neovascularisation, and reduce wound contraction and scar formation [89]. The material should certainly not be degraded by the AF and bidirectional impermeable to both AF and CSF. The scaffold must be easy to handle and to apply, also in an endoscopic approach. The scaffold must protect the spinal cord against intrauterine chemical and

mechanical injury without the formation of adhesions between neural tissue and skin with consequently tethering of the cord.

The scaffolds we used in our experiments were developed at the Department of Biochemistry, University Medical Center Nijmegen, The Netherlands and fashioned from the natural material, type I collagen [88-90]. One of the major advantages of this scaffold is that it has predefined physico-chemical, biomechanical, and morphological characteristics and can be modulated in different ways to comply with all the requirements for use in fetal therapy [90-92]. For instance, cell migration can be controlled by modifying the matrix structure and the incorporation of glycosaminoglycans and growth factors. In addition, vascular ingrowth can be enhanced and consequently, scar formation reduced [87-89,93-95]. Angiogenesis is especially important to generate sufficient oxygen and nutrients supply in constructs with large surface area to prevent central necrosis. By stimulating tissue ingrowth and neovascularisation, the scaffold will ultimately be replaced by native surrounding tissue.
Although tissue engineering techniques offer good prospects for use in fetal therapy, before commencement of clinical trials and application of tissue-engineered constructs in the human fetus, ethical issues still need to be addressed [96].

THESIS 'FETAL THERAPY FOR SPINA BIFIDA IN A SHEEP MODEL USING TISSUE ENGINEERING'

The aims of the thesis were:

- To evaluate whether *acute* intrauterine coverage of an experimental, surgically created, neural tube defect in fetal lambs can protect neural tissue from secondary injury during gestation, improve histological outcome and save neurologic functions after birth.
- To evaluate whether a collagen scaffold is useful for *delayed* in utero repair of a surgically created spina bifida in the fetal lamb and can protect the spinal cord from secondary injury.
- To investigate whether a collagen scaffold loaded with growth factors can be used to treat full-thickness fetal skin defects.
- To evaluate the effect of amniotic fluid exchange on the concentrations of waste products in the amniotic fluid and to assess the histological outcome in fetal sheep with a surgically created neural tube defect.
- To provide an overview of adaptations and refinements of the protocols of the fetal sheep model for congenital birth defects to reduce the use of laboratory animals and to improve animal welfare.

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CHAPTER 2

In utero repair of an experimental neural tube defect in a chronic sheep model using biomatrices

authors
A.J. Eggink
L.A.J. Roelofs
W.F.J. Feitz
R.M.H. Wijnen
R.A. Mullaart
J.A. Grotenhuis
T.H. van Kuppevelt
M.M.Y. Lammens
A.J. Crevels
A. Hanssen
P.P. van den Berg

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Abstract

Objective: Persistent exposure of the unprotected spinal cord to amniotic fluid and the uterine wall can lead to progressive damage of neural tissue in case of a myelomeningocele ('two-hit' hypothesis). The aim of this study was to evaluate whether in utero repair of an experimental neural tube defect in a fetal lamb could protect neural tissue from secondary injury and save neurologic functions after birth.

Methods: In 19 fetal lambs, a neural tube defect was created at 79 days' gestation. In 12 lambs the defect was covered either with a novel, molecular defined collagen-based biocompatible and biodegradable matrix (UMC) or with a small intestinal submucosa (SIS) biomatrix (Cook®) or by closing the skin over the defect.

Results: All lambs with the defect covered showed no or minor neurologic morbidity in contrast to the lambs with the defect uncovered in which major neurologic morbidity was seen.

Conclusions: These results demonstrate that long-term exposure of the open spinal cord to the intrauterine environment can lead to damage of neural tissue and, consequently loss of neurologic functions and that coverage of the defect can lead to a better neurologic outcome. Furthermore, we could show that a UMC biomatrix and an SIS biomatrix are useful for in utero coverage of a surgically created neural tube defect in our model.

Introduction

Myelomeningocele is one of the most common birth defects involving the central nervous system. It occurs in approximately 1 of every 2,000 live births and can lead to neurologic injury above and below the level of the lesion such as hydrocephalus, hindbrain herniation, mental impairment, paraplegia, urinary and fecal incontinence, sexual dysfunction and skeletal deformities.

The etiology of myelomeningocele is usually unknown and most likely multifactorial in which both genetic and environmental factors (e.g. nutrition, vitamin deficiencies) play a role [1,2].

The pathogenic mechanism is either a primary disorder of neurulation with failure of the neural plate to close or failure of mesenchymal closure at the caudal neuropore in the embryonic period (first-hit). Consequently, due to the absence of the musculoskeletal coverage, the persistent exposure of the unprotected spinal cord to amniotic fluid and the uterine wall can lead to progressive mechanical and/or chemical damage ('two-hit' hypothesis) [3-6]. This secondary injury to the exposed neural tissue could be a main factor causing loss of neurological function. In utero repair of a myelomeningocele might arrest this process of progressive neural tissue destruction and rescue neurologic functions at birth [7,8].

The aim of this study was to evaluate whether in utero coverage of an experimental neural tube defect in a fetal lamb with a novel, molecular defined collagen-based biocompatible and biodegradable matrix (UMC biomatrix), a small intestinal submucosa (SIS) biomatrix (Cook®) or by closing the skin over the defect could protect neural tissue from secondary injury and save neurologic functions after birth.

Materials and methods

Animals

After obtaining approval from the Ethical Committee on Animal Research, pregnant sheep (Dutch Texel breed) were operated in this study at 79 days' gestation (full term, 140-147 days). Anesthesia for surgery was induced by an intravenous injection of 20-25 ml pentobarbital (60 mg/ml) and 0.5 ml atropine (0.5 mg/ml) and, following endotracheal intubation, maintained with 2% isoflurane and O_2/N_2O ventilation with a respiration rate of 16/min. The uterus was exteriorized through a midline abdominal incision under sterile conditions and inspected for the number of fetuses. At a favorable location a hysterotomy was performed and the hindlimbs and back of the fetus were exposed. The uterus and fetus were wrapped in a warm saline-soaked towel.

Preparation of collagen matrices

The SIS biomatrix was obtained from COOK® Biotechnology (Bloomington, Ind., USA). Biochemically defined collagen-based matrices were made from bovine tendon by the Department of Biochemistry, UMC Nijmegen, The Netherlands. This procedure was described previously in detail by Pieper et al. [9].

In short, collagen suspensions were prepared by incubation of 0.8% insoluble type I collagen in 0.5 *M* HAc (pH 2.5) for 16 h at 4 °C. Suspensions were homogenized at 4 °C. The collagen suspension was de-aerated under vacuum to remove entrapped air bubbles. Collagen suspension was poured into polystyrene culture flasks, quickly frozen at -80 °C and lyophilized, resulting in porous matrices with a layer thickness of 5 mm.

Chemical cross-linking of collagen matrices was performed using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS). Collagen matrices of 50 mg dry weight were incubated for 30 min in 20 ml of 50 m*M* 2-morpholinoethanesulfonic acid (MES) (pH 5.5) containing 40% (v/v) ethanol. Subsequently, the matrices were cross-linked by immersion in 19.5 of 50 m*M* MES (pH 5.5) containing 33 m*M* EDC and 6 m*M* NHS, containing 40% (v/v) ethanol. After reaction for 4 h, the matrices were washed twice in 0.1 *M* Na_2HPO_4 (pH 9.1) for 1 h. Finally, the matrices were washed with 1 *M* NaCl for 2 h and with 2 *M* NaCl for 1 day (with 6 changes of washing solution), followed by washing with distilled water and lyophilization.

Before implantation the matrices were washed in 70% (v/v) ethanol (4 x 30 min) and sterile phosphate-buffered saline (pH 7.4) (5 x 15 min) at 20 °C.

Surgical Procedure

The animals were divided into 5 groups. In the first group (Sham control) 3 skin lesions, approximately 1.5 x 1.5 cm, were made, 1 on each buttock and 1 in the middle of the lower back (fig. 1). The lesion on the left buttock was covered with the defined collagen UMC biomatrix. On the right side, the lesion was covered with the SIS biomatrix. The third lesion in the middle was left open.

In the other 4 groups, a neural tube defect was created surgically by excising skin and paraspinal muscles and soft tissue and performing a laminectomy of 3 lumbar vertebrae (L3-5). The outward flow of cerebrospinal fluid could be confirmed after opening of the dura in the midline over the length of the 3 vertebrae. The lesions measured approximately 2 x 3 cm, as shown in figure 2. In the second group, the above-described lesion was left uncovered so that the neural tissue remained exposed to the amniotic fluid. In the other groups, the defect was immediately covered during the same operation. In the third group, the lesion was covered with the defined collagen UMC biomatrix. In the fourth group, the defect was closed by closing the skin with 6-0 monocryl interrupted sutures. In the fifth group, the SIS biomatrix was used to cover the defect. Both biomatrices were sutured to the surrounding tissue with eight 6-0 prolene interrupted sutures. After the surgical procedure the fetus was replaced in the uterus and amniotic fluid volume was restored with warm sterile saline. 250 mg of amoxicilline was instilled into the amniotic fluid and the uterus was closed with a running suture in 2 layers using 2-0 vicryl. Natrium Penicillium (1,000,000 IU) was given in the intra-abdominal space and the maternal laparotomy was closed in layers using 1-0 vicryl. Depomycin (20 mg/kg, s.c.) was initiated preoperatively and maintained postoperatively for 3 days. The animals stayed for 3-5 days at the Central Animal Laboratory of the University of Nijmegen and were fed normally before returning to the farm.

Neonatal Outcome

At 140 days' gestation, the lambs were delivered by cesarean section under local anesthesia with 20-30 ml 2% lidocaine and were observed for 1 week. A neurological examination was carried out between day 2 and 7 of life according to a standardized protocol including spontaneous and elicited behavior, with special emphasis on motor, sensory and reflex modalities of hind- and forelimbs, anal sphincter and urinary bladder. Experimental and control lambs were videotaped and photographed to enable comparison. The findings were expressed as the upper level of loss of spinal function (S5 means no

loss of function). Continual urine loss and urine loss on bladder expression were taken as criteria of incontinence. Afterwards the lambs were sacrificed with xylazine (10 mg given intramuscular) and pentobarbital (60 mg/kg given intracardiac) and macroscopic and microscopic evaluation of the lesions were performed by a single experienced neuropathologist. Brain and spinal cord were examined for the presence of hindbrain herniation or hydrocephalus. Hindbrain herniation was defined as the descent of the cerebellar vermis and brainstem below the level of the foramen magnum.

Results

Surgery was performed in 23 fetal lambs. Survival rates are shown in table 1. Three lambs were aborted after fetal surgery, 1 lamb died during the cesarean section and 3 lambs died at the first day of life. The overall fetal survival rate was 87% during the pregnancy period. No maternal deaths occurred in the study period.

The first group was used as sham control to assess wound healing in utero and to evaluate the reaction of the skin and underlying tissues in the used biomatrices. Table 2 describes the wound healing in this group. On macroscopic inspection, the skin covering the biomatrices was closed in all lambs. Above the lesion covered with the UMC biomatrix the fleece was thin or not

TABLE 1. SURVIVAL RATES

Group	Operated	Aborted	Died postpartum	Survived
1. Sham control	4	0	2	2
2. NTD, uncovered	7	1	1	5
3. NTD, UMC biomatrix	4	1	1	2
4. NTD, muscle / skin	3	0	0	3
5. NTD, SIS biomatrix	5	1	0	4
Total	23	3	4	16

NTD = Surgically created neural tube defect.

present at all in 3 of the 4 lambs. The skin covering the SIS biomatrix showed a normal fleece pattern although the biomatrix had a tendency to shrink in these lambs. On microscopic examination, the biomatrices were covered with scar tissue and seemed to be well integrated in the subcutaneous tissue. They were partially broken down and there was some ingrowth of fibroblasts from the surrounding tissue. Histologically the SIS biomatrix and the UMC biomatrix were comparable.

A neural tube defect was created surgically in 19 fetal lambs. Three of them aborted and these could not be further evaluated. Wound healing, neurologic outcome and function of the urinary bladder are described in table 3. Two neurologically impaired lambs produced solid stools, all others smooth stools. On macroscopic inspection the skin covering the defect was normally healed in 11 lambs. In the other 5 lambs a small or large defect was present as shown in figure 3; 3 of them were within the group with the neural tube defect uncovered (second group). All lambs with a covered neural tube defect (third, fourth and fifth group) showed no or minor loss of spinal function. Four of the five lambs with the neural tube defect uncovered (second group) had paraplegia and sphincter paresis. Only 1 lamb within this group showed no neuromorbidity. Comparing these groups, there was a statistically significant difference in neurologic outcome between the group with the defect uncovered and the groups with the defect covered ($p=0.017$). The differences within the group with the neural tube defect covered (either with the bioma-

TABLE 2. WOUND HEALING IN THE FIRST GROUP

Lamb No.	Skin lesion uncovered	Skin lesion covered with UMC biomatrix	Skin lesion covered with SIS biomatrix
1	skin closed	skin closed, no fleece	skin closed
2	skin closed	skin closed	skin closed
3	skin closed	skin closed, no fleece	skin closed
4	skin closed, thin fleece	skin closed, thin fleece	skin closed, thin fleece

TABLE 3. WOUND HEALING, NEUROLOGIC OUTCOME AND URINARY CONTINENCE IN LAMBS WITH A SURGICALLY CREATED NEURAL TUBE DEFECT

Group		Macroscopic appearance of the defect	Level of spinal dysfunction	Function of the urinary bladder
NTD uncovered	1	closed	L1	incontinent
	2	closed	C4	incontinent
	3*	closed	X***	X***
	4	small defect	S5	continent
	5	large defect	L1	incontinent
	6	large defect	C4	incontinent
NTD closure	1*	closed	X***	X***
UMC biomatrix	2	large defect	S5	continent
	3	closed/no fleece	S5	continent
NTD closure	1**	closed	X***	continent
skin	2	closed	S5	continent
	3	closed	S5	continent
NTD closure	1	closed	S5	continent
SIS biomatrix	2	closed/no fleece	S5	continent
	3	closed/no fleece	S5	continent
	4	small defect	S5	continent

* Died postpartum; ** no neurological examination was carried out; *** no values. S5 means no loss of function.
The difference in neurologic outcome between the group with the defect covered and the groups with the defect uncovered is statistically significant ($p = 0.017$).

trices or with skin) were small. This applied for neurologic outcome as well as for superficial wound healing. There were no signs of hydrocephalus or hindbrain herniation on macroscopic inspection of the brain and spinal cord.

Discussion

The theoretical basis for in utero repair of a myelomeningocele is the 'two-hit' hypothesis. A primary failure in the embryonic period leads to the development of myelodysplasia (first-hit) and consequently, due to the absence of the skin and musculoskeletal coverage, the persistent exposure of the unprotected spinal cord to the intrauterine environment can lead to secondary destruction of neural tissue (second-hit) [5,7]. In utero repair of a myelomeningocele may improve neurologic outcome by preventing at least part of the secondary damage.

This hypothesis has been further developed in several animal experiments in which an experimental neural tube defect was created in early pregnancy. Meuli et al. [3] showed in fetal sheep that exposure of the normal and undamaged spinal cord to the intrauterine environment leads to pathology that has clinical and morphological similarities to a human meningomyelocele. Paek et al. [4] found in a similar fetal lamb model, that prenatal repair prevents or reverses development of cerebral co-morbidity, in particular of hindbrain herniation. Michejda et al. [8] found that in utero coverage of an experimental spina-bifida-like lesion in a primate model with allogeneic bone paste leads to a normal neurological condition at birth.

In this study we could show that creation and covering of an experimental neural tube defect in a chronic sheep model is feasible. However, a classical human like dysraphic lesion, such as the myelomeningocele, could not be created nor did we find secondary morbidity such as hydrocephalus or hindbrain herniation. Four of five neurologically examined lambs with the defect uncovered showed major neurologic impairment while all lambs with the defect covered were unimpaired. The latter implies that the operation itself cannot be the cause of the neurologic impairment, but instead the long-term exposure of the spinal cord to the intrauterine environment (second-hit) might be the cause, as was shown in earlier studies. However, it should be noted that spontaneous closure of the defect was found in 3 of the 6 lambs with the defect uncovered. Maybe the created defect was too small so that spontaneous partial healing could occur. In spite of the fact that it is unknown as to how long the duration of exposure to the intrauterine environment must be

for neurologic injury to occur, in view of the observed neurologic morbidity it was long enough to cause damage to the neural tissue. In a fetal rat model Heffez et al. [5] found signs of extensive spinal cord injury 48 h after creating a surgically simulated spinal dysraphism. In this study we covered the defect immediately after creation. To simulate the untreated period of the human condition, coverage of the experimental defect should be delayed. However, to answer the question whether biomatrices are useful in intrauterine repair of an experimental neural tube defect and can protect the spinal cord for further damage, delayed coverage is not necessary.

Various techniques, such as closure of the defect with an acellular skin matrix, latissimus dorsi muscle flap, maternal split thickness skin graft or neurosurgical closure in utero with dura have been used [4,7,10-13]. In this model we evaluated a novel molecularly defined UMC biomatrix and compared this with an SIS biomatrix, a xenogenic membrane that is composed mainly of the submucosal layer of the intestinal wall harvested from porcine small intestine. These biomatrices have been used earlier, especially for urogenital reconstructions in animals [14,15]. In this study we used the simplest defined biomatrix and the results of the UMC biomatrix were comparable to the SIS. We evaluated fetal wound healing in utero and the reaction of the skin and underlying tissues to the used biomatrices and could show that they can be useful for in utero coverage of a surgically created neural tube defect. The major advantage of defined biomatrices in comparison to SIS is that they can be modulated in different ways, e.g. incorporation of factors to promote cell growth. Development of biological substitutes designed to maintain, restore or improve tissue function, making use of biocompatible and biodegradable materials can have essential advantages in the field of tissue engineering.

In humans, intrauterine myelomeningocele repair appears to result in a significant reduction in shunt-dependent hydrocephalus and may reduce the degree of hindbrain herniation, however, the effects on the sensorimotor functions are disappointing [16-19]. At this moment, the benefits of intrauterine myelomeningocele repair might not outweigh the fetal and maternal risks of intrauterine surgery (premature delivery, operative complications and rupture of the uterus) outside closely monitored experimental studies. To reduce risks of intrauterine repair, further research should be performed to improve minimal invasive surgical techniques (e.g. endoscopic) for fetal surgery in which tissue-engineering methods might play a role [20,21]. The use of biomatrices offers good prospects for the endoscopic approach.

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CHAPTER 3

Histological evaluation of acute covering of an experimental neural tube defect with biomatrices in fetal sheep

authors
A.J. Eggink
L.A.J. Roelofs
M.M.Y. Lammens
W.F.J. Feitz
R.M.H. Wijnen
R.A. Mullaart
H.T.B. van Moerkerk
T.H. van Kuppevelt
A.J. Crevels
A. Hanssen
F.K. Lotgering
P.P. van den Berg

Fetal Diagnosis and Therapy 2006;21:210-216

Abstract

Objective: The aim of the study was to determine the histological effect on the neural tissue of in utero covering of an experimental neural tube defect in fetal lambs, with the use of two different biomatrices.
Materials and Methods: In 23 fetal sheep, surgery was performed at 79 days' gestation. In 19 of these, a neural tube defect was created, while 4 fetuses served as sham-operated controls. In 7 of the 19 operated fetuses the defect was left uncovered. In the remaining 12 animals the defect was covered either with a collagen biomatrix (4 animals), skin (3 animals) or small intestinal submucosa biomatrix (5 animals). The lambs were sacrificed at 1 week of age and histological examination was performed.
Results: All lambs with an uncovered neural tube defect showed histological damage of the spinal cord. In lambs in which the neural tube defect was covered, one half showed a normal architecture of the spinal cord while minor histological damage was present in the other half. Between the three groups in which the defect was covered, the histological outcome was comparable.
Conclusions: Acute covering of an experimental neural tube defect in fetal lambs prevents severe histological damage to the spinal cord independent of the two biomatrices used in this study.

Introduction

Spina bifida aperta (SBA) can result in lifelong impairment of the lower extremities, fecal and urinary incontinence, sexual dysfunction, and mental impairment. In most cases hindbrain herniation and hydrocephalus are present. Because of the severe morbidity, lifelong supportive care is often needed. SBA has a worldwide incidence of about 1 per 2,000 live births.

Due to a maldevelopment of the neural plate in the embryonic period, secondary injury to the unprotected spinal cord resulting from local trauma or from amniotic fluid toxicity, can lead to additional neural damage [1,2]. The secondary damage may be prevented or even reversed by early fetal intervention.

The hypothesis that in utero repair of an SBA may preserve neurological function has been demonstrated in animal experiments [3-7]. We previously reported that covering of an experimental defect can lead to a clinically better neurologic outcome [8]. Currently, no robust scientific evidence proves that fetal surgery for SBA has a benefit for the postnatal functions of legs, bladder and bowel of human fetuses. However, it has been suggested that intrauterine SBA repair in humans may result in reduction of the degree of hindbrain herniation and a reduction in the need for postnatal ventricular shunting [9-13]. Open fetal surgery is very invasive for the mother, as it requires maternal laparotomy and hysterotomy. In order to decrease the maternal trauma from fetal surgery, less invasive access methods have been investigated [14-16]. Because it is difficult to close a neural tube defect endoscopically with the use of current techniques, the endoscopic approach demands further research into materials to cover the defect. New covering materials should be equally effective as standard neurosurgical repair and should have no adverse effects on the neural tissues, including tethering of the spinal cord.

The aim of the study was to determine the histological effect on the neural tissue of acute in utero covering of an experimental neural tube defect in fetal lambs with the use of two different biomatrices and to study the incorporation of the biomatrix in the surrounding tissues. The outcome was compared to covering the defect with skin or leaving the defect exposed.

Materials and Methods

Biomatrices

For the purpose of this study we used two different biomatrices; a commercially available small intestinal submucosa (SIS) biomatrix obtained from COOK Biotechnology (Bloomington, Ind., USA) and a molecular defined collagen-based biocompatible and -degradable biomatrix (UMC biomatrix) developed by the Department of Biochemistry of the Nijmegen Centre for Molecular Life Sciences (Radboud University Nijmegen Medical Centre, The Netherlands). This matrix is made from insoluble type I collagen, isolated from bovine Achilles tendon as previously described in detail [8,17].

Surgical procedure

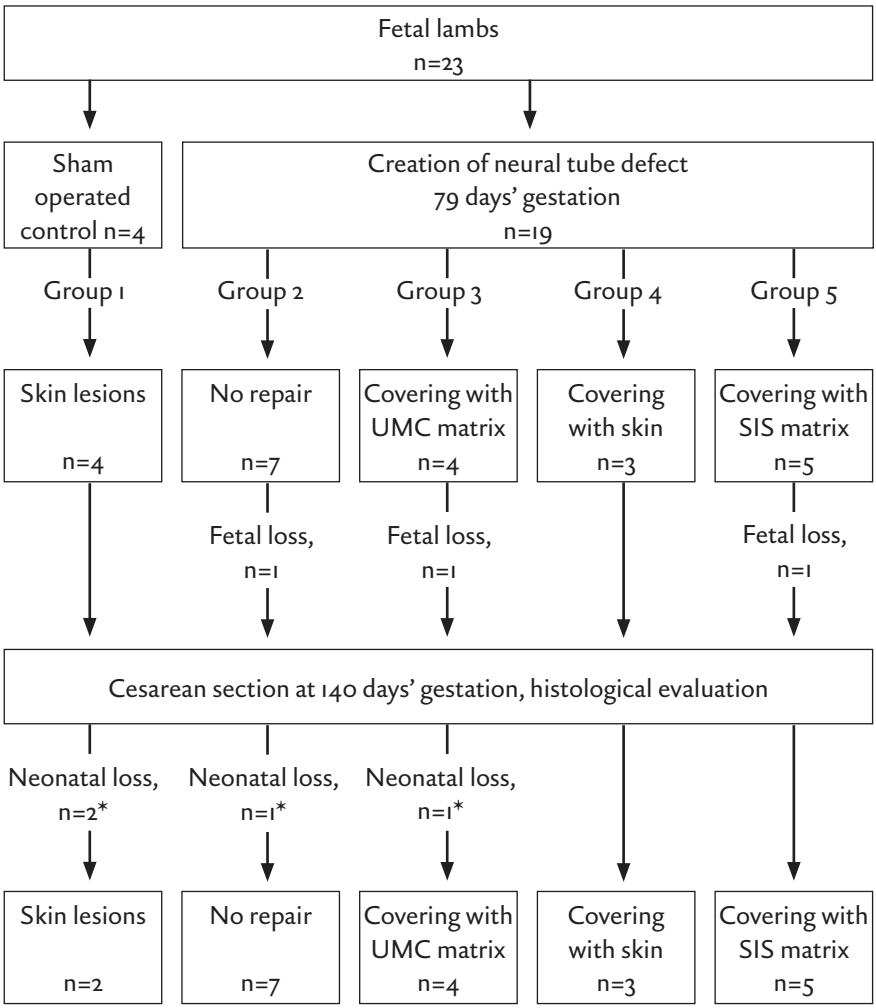
A total of 23 pregnant sheep of Dutch Texel breed underwent surgery at 79 days' gestation (term, 140-147 days). The protocol had been approved by the Ethical Committee on Animal Research of the Radboud University Nijmegen, The Netherlands.

The animals were premedicated with an intravenous injection of pentobarbital (20-25 ml; 60 mg/ml) and atropine (0.5 ml; 0.5 mg/ml) and underwent general endotracheal anesthesia with 2% isoflurane and O_2/N_2O ventilation. Under sterile conditions, the uterus was exteriorized through a lower midline laparotomy and the position of the fetal lamb was identified by palpation. A hysterotomy was performed and the hindlimbs and back of the fetus were exteriorized. The exteriorized uterine horn and fetus were wrapped in warm saline-soaked towels.

The animals were divided into 5 surgical groups, as represented in figure 1. Group 1 consisted of sham-operated controls in which 3 skin lesions of approximately 1.5 x 1.5 cm, were made, one on each buttock and one in the middle of the lower back. The lesion on the left buttock was covered with the UMC biomatrix, on the right side, the lesion was covered with the SIS biomatrix, and the lesion in the middle was left open.

In the other 4 groups, a neural tube defect was surgically created. The skin, paraspinal muscles and soft tissue were removed and a laminectomy was performed at L3-L5. The dura was opened in the midline over the length of the 3 vertebrae and the outward flow of cerebrospinal fluid was confirmed. The lesions measured approximately 2 x 3 cm. In group 2 the lesion was left uncovered with the neural tissue exposed to the amniotic fluid. In the other three groups the defect was covered immediately in the same session. In group 3, the lesion was covered with the UMC biomatrix, in group 4 with skin, and in

FIG. 1. SCHEDULE OF EXPERIMENTS AND SURVIVAL



* Still available for histological evaluation.

group 5 with the SIS biomatrix. Both biomatrices were sutured to the surrounding tissue using eight 6-0 prolene interrupted sutures, while for the skin 6-0 monocryl interrupted sutures were used. The fetus was replaced in the uterus and amniotic fluid volume was restored with warm saline solution. Amoxicilline (250 mg) was added to the amniotic fluid. The uterus was closed with a running 2-0 vicryl suture in two layers. Sodium penicillin (1,000,000 IU) was administered to the intra-abdominal space and the abdominal wall was closed in layers using 1-0 vicryl. Depomycine (20 mg/kg, s.c.) was initi-

ated preoperatively and maintained postoperatively for 3 days. The animals stayed for 3 - 5 days at the Central Animal Laboratory of the University of Nijmegen and were fed normally before they were returned to the farm.

Histology

At 140 days' gestation, the lambs were delivered by cesarean section under local anesthesia with lidocaine (20-30 ml; 2%) and were observed for 1 week. A neurological examination was carried out between day 2 and 7 of life according to a standardized protocol. The findings were expressed as the upper level of loss of spinal function (S5 means no loss of function). At 1 week of age, the lambs were sacrificed with an intramuscular injection of xylazine (10 mg) followed by intracardial injection of pentobarbital (60 mg/kg). In the sham-operated control group, histological sections of the skin and subcutaneous tissues were made at the level of the skin lesions. In the other groups the vertebral column, spinal cord and brain were excised and fixated in formaldehyde 4%. The skull and cervical vertebral column were opened and examined for the presence of hydrocephalus and hindbrain herniation, defined as a downward displacement through the foramen magnum of the cerebellar vermis and medulla. Serial cross-sections of the spinal canal and cord with the surrounding tissues were taken at the lumbosacral level and included the site of surgery. Further cross-sections of the spinal cord were taken at the thoracic and cervical levels. The histological slides were stained with hematoxylin/eosin and luxol fastblue/hematoxylin. They were studied by light microscopy by a single experienced neuropathologist.

Results

The fetal survival rate during the pregnancy period was 87%. Three lambs aborted, 1 lamb died during the cesarean section and 3 lambs died on the 1st day of life. The outcomes of the neurological assessment have been previously reported [8]. Briefly, all lambs with a covered neural tube defect showed either no or minor loss of spinal function, while 4 of the 5 lambs with the neural tube defect uncovered had paraplegia and demonstrated urinary incontinence.

On macroscopic inspection, in all sham-operated control lambs (group 1), the skin covering the biomatrices was closed. In group 2 (neural tube defect uncovered), in 3 of the 6 lambs the skin had closed spontaneously while in the other three lambs a skin defect was present. In group 3 (neural tube defect covered with UMC biomatrix), 1 lamb showed a large defect of the skin while

in the other 2 lambs the skin had closed. In group 4 (neural tube defect closed with skin), normal wound healing had occurred. In group 5 (neural tube defect covered with SIS biomatrix), 1 lamb showed a small defect of the skin while in the other 3 lambs the skin had closed.
The major histological findings are listed in table 1. Figure 2 shows an example of a normal spinal cord. In group 1 (sham-operated controls), in all 4 lambs

TABLE 1. NEUROLOGICAL OUTCOME AND MICROSCOPICAL FINDINGS IN LAMBS WITH A SURGICALLY CREATED NEURAL TUBE DEFECT (NTD)

Group	Animal	Level of spinal dysfunction	Microscopic appearance of the spinal cord
Group 2	1	L1	neuronal migration disturbances
NTD	2	C4	unilateral syringomyelia and focal hydromyelia
uncovered	3*	X	hydromyelia
	4	S5	syringomyelia
	5	L1	meningomyelocele
	6	C4	meningomyelocele
Group 3	1*	X	neuronal migration disturbances
NTD closure	2	S5	normal
UMC biomatrix	3	S5	normal
Group 4	1**	X	hydromyelia and syringomyelia
NTD closure	2	S5	normal
skin	3	S5	normal
Group 5	1	S5	neuronal migration disturbances
NTD closure	2	S5	minor defect central canal
SIS biomatrix	3	S5	normal
	4	S5	minor defect central canal

* Died postpartum; ** no neurological examination was performed. X no values. S5 means no loss of function.

the biomatrices had been well incorporated into the surrounding tissues. The matrices had been partially broken down and there was some ingrowth of fibroblasts from the surrounding tissues. In group 2 (neural tube defect uncovered), all 6 lambs showed histological abnormalities of the spinal cord. One lamb showed neuronal migration disturbances in which a few neurons or groups of neurons were found in anatomically abnormal locations in the white matter. Three of the 6 lambs in this group showed syringo- or hydromyelia (fig. 3) and 2 lambs an SBA with severe damage of the exposed dorsal part of the cord (fig. 4,5). The spinal cord of these 2 lambs had been split and the dorsal horns had merged with the skin. Both lambs showed major neurological impairment. In group 3 (neural tube defect covered with UMC biomatrix), 1 lamb showed neuronal migration disturbances similar to that in group 2. In the other 2 lambs the normal architecture of the spinal cord was preserved. In group 4 (neural tube defect closed with skin), 1 lamb showed hydro- and syringomyelia. In the other 2 lambs no damage of the spinal cord was found. In group 5 (neural tube defect covered with SIS biomatrix), 1 lamb showed neuronal migration disturbances and 2 lambs showed a minor defect of the ependymal lining of the central canal. In 1 lamb no damage of the spinal cord was found. Figure 6 shows examples of incorporation of the biomatrices into the surrounding tissues.
In all groups, hydrocephalus or hindbrain herniation could not be found on pathological examination. Signs of direct trauma due to the operation itself or to abrasion of the neural tissue with the uterine wall or an inflammatory reaction were not found. In all three groups with the neural tube defect covered, ingrowth of adjacent structures into the neural tissue was not found.

Discussion

All lambs with the defect uncovered showed histological abnormalities of the spinal cord. Neither signs of direct trauma due to the operation itself or recent trauma due to contact with the uterine wall, nor signs of an inflammatory reaction were found. This suggest that long-term exposure of an unprotected spinal cord to the intrauterine environment, more specifically the amniotic fluid, can lead to severe damage of the spinal cord ('two-hit' hypothesis) and that acute covering of a surgically created neural tube defect can preserve the architecture of the spinal cord [3,5,18].
We used a novel, molecularly defined, collagen-based, biocompatible and biodegradable biomatrix and compared it, because of earlier experience in an

animal model, with an SIS biomatrix [19]. The UMC biomatrix is made from insoluble type I collagen and after preparation the isolated collagen is free of other proteins. A physical method of cross-linking has stabilized the collagen and prevents collapse of the matrix. In vitro evaluation has confirmed that the matrices do not introduce cytotoxicity [17]. The pore diameters of the biomatrices that were used ranged from 50 to 100 μm. It has been hypothesized that due to its biodegradability the biomatrix will degrade and ultimately be replaced by native collagen. The main advantage of the UMC biomatrix is that it can be modulated in various ways. The porous matrix structure can be altered or growth factors can be incorporated to enhance wound healing. In this study, the simplest defined UMC biomatrix was used. The SIS biomatrix is a xenogenic membrane composed mainly of the submucosal layer of the porcine small intestine, from which mucosa, serosa and tunica muscularis have mechanically been removed from the inner and outer surfaces of the graft.

The resemblance in histological findings between both biomatrices is in accordance with a previous study [19]. Both biomatrices were incorporated well into the surrounding tissues and ingrowth of fibroblasts was demonstrated. In all groups with the defect covered, the architecture of the spinal cord was mainly well preserved although neuronal migration disturbances were not found in the group in which the defect was covered by closing the skin. The observation of neuronal migration disturbances, as observed in both the uncovered and the covered group, has not been previously reported and remains to be elucidated. Just as relevant is our observation that covering of the defect with biomatrices is not accompanied by ingrowth of the spinal cord by adjacent structures. As long as the spinal cord keeps free from its surroundings, the risk of tethered cord is smaller. However in the human situation, the need for postnatal repair would still be necessary because the rostral and caudal ends of the neural placode remain attached to surrounding tissues but may be postponed beyond the neonatal period. The biomatrix itself would not have to be removed postnatally because it is biodegradable. The absence of adhesions between the spinal cord and patch material lowers the risk of functionally relevant damage to the cord during postnatal repair. Follow-up was too short to exlude the formation of adhesions in the long-term. In 3 of the 6 lambs with the defect uncovered spontaneous closure of the skin occurred. Possibly the created defect was too small but it could also be attributed to the strong healing potential of fetal tissues at this early gestation. In view of the histological findings the duration of exposure to the intrauterine environment was long enough to cause some damage to the

spinal cord; neuronal migration disturbances, syringo- or hydromyelia were found in these 3 cases.

This animal model seems to be suitable to investigate covering materials and techniques for fetal SBA repair; however, it does not exactly replicate the human malformation. Although in 2 lambs, the created defect remained open, the associated anomalies such as hydrocephalus or hindbrain herniation, as reported by Paek et al. [5] and Bouchard et al. [20], did not occur. However, in contrast to our study, the surgically created lesion in both studies referred to included also a myelotomy. Another difference with the natural spina bifida is that the surgical defect is artificially created in midgestation and exists for a shorter period of time before eventual repair. To simulate the untreated period of a human spina bifida, repair of the experimental defect should be delayed. For the aim of this present study, delayed covering was not essential.

In conclusion, covering of an experimental neural tube defect can preserve the architecture of the spinal cord and consequently, intrauterine repair might lead to a better neurological outcome. Difference in histological outcome between the methods used to close the defect could not be demonstrated.

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CHAPTER 4

Delayed intrauterine repair of an experimental spina bifida with a collagen biomatrix

authors
A.J. Eggink
L.A.J. Roelofs
W.F.J. Feitz
R.M.H. Wijnen
M.M.Y. Lammens
R.A. Mullaart
H.T.B. van Moerkerk
T.H. van Kuppevelt
A.J. Crevels
K. Verrijp
F.K. Lotgering
P.P. van den Berg

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Abstract

Background/Purpose: The aim of the study was to evaluate whether a collagen biomatrix is useful for delayed intrauterine coverage of a surgically created spina bifida in a fetal lamb.
Methods: In 20 fetal lambs, surgery was performed at 72 or 79 days' gestation. In 15 lambs a spina bifida was created surgically. In 8 lambs it was covered with a collagen biomatrix 2 weeks later and in 7 lambs it was left uncovered. Five lambs served as sham operated controls. Neurological examination was performed at 1 week of age and afterwards the lambs were sacrificed for further histological evaluation.
Results: None of the 5 surviving lambs with the defect covered showed loss of spinal function and the architecture of the spinal cord was preserved in 4 of the 5 lambs. In the uncovered group, 1 of the 4 surviving lambs had loss of spinal function, 5 lambs were available for histological evaluation and 4 of them showed disturbance of the architecture of the spinal cord.
Conclusions: Collagen biomatrices can be used for intrauterine coverage of an experimental spina bifida and can preserve the architecture of the spinal cord. Neurological outcome is not different between fetuses with their spinal cord covered and fetuses with uncovered cords.

Introduction

The first in utero repair of a surgically created spina bifida-like lesion in an experimental animal model was done in 1984 [1]. Since then, many animal studies have described the intrauterine fetal repair of spina bifida aperta [2-9]. The theoretical basis for in utero repair of a spina bifida aperta is the 'two-hit' hypothesis. Intrauterine coverage of the unprotected spinal cord may prevent the neural damage (second hit) to the spinal cord caused by the intrauterine environment. The first in utero spina bifida repair of a human fetus was performed in 1994 and since then more than 200 in utero spina bifida closures have been done [10-12]. Various materials, for example, Alloderm, split or full-thickness skin graft, allogeneic bone paste or Xenoderm, have been used for in utero coverage of the spinal cord [1,6,7,9,13-16]. Intrauterine repair may result in reduction of the degree of hindbrain herniation and the need for postnatal ventricular shunting. Benefits for the postnatal functions of legs, bladder and bowel have not yet been proven [13,16-21].
The aim of this study was to evaluate to what extent a collagen biomatrix is useful for the delayed coverage of a surgically created spina bifida aperta in the fetal lamb and whether it can protect the developing spinal cord from secondary injury.

Materials and methods

The study was approved by the local committee for experiments on animals of the Radboud University Nijmegen, The Netherlands.

Biomatrices

The biomatrix used in this study was made from type I collagen, developed by and manufactured at the Department of Biochemistry of the Nijmegen Centre for Molecular Life Sciences (Radboud University Nijmegen Medical Centre, The Netherlands). The collagen biomatrix was isolated from bovine Achilles tendon as previously described [22;23].

Surgical procedure

Twenty pregnant sheep underwent their first operation at 72 or 79 days' gestation. (Dutch Texel breed; term 140-147 days). The animals were premedicated with an intravenous injection of pentobarbital (17-45 ml; 60 mg/ml) and atropine (1 ml; 0.5 mg/ml) and were maintained on general anesthesia with 2% isoflurane and O_2/N_2O ventilation. Fetal anesthesia was achieved by transplacental passage of the medications, administered to the mother. After a low midline laparotomy under sterile conditions, one horn of the uterus was partially mobilized out of the abdominal cavity. The number and position of the fetal lambs was determined by palpation. For fetal surgery, the back and the hindlimbs of the fetus were exposed through a uterine incision and the fetus and uterus were wrapped in a warm saline-soaked towel.

The animals were divided into 3 groups. In group 1 and 2, an experimental surgical spina bifida aperta was created in the fetal lamb by excision of the skin (approximately 3 cm in diameter), paraspinal muscles and soft tissue and performing a laminectomy of 3 lumbar vertebrae (L_{3-5}). After opening of the dura in the midline over the length of the 3 vertebrae, the outward flow of liquor was confirmed in each case. In both groups, the lesions were left uncovered. Four 5-0 prolene interrupted sutures were placed to mark the defect. Group 3 served as sham-operated controls. Two skin lesions, approximately 1.5 x 1.5 cm, one on each buttock, were made. The lesion on the right side was covered with the biomatrix which was secured into place using four 5-0 prolene interrupted sutures. The lesion on the left side was left uncovered. To mark the defect, four 5-0 prolene interrupted sutures were placed around each skin lesion. After surgery the fetus was returned to the uterus. Amniotic fluid volume was restored with warm sterile saline solution and amoxicillin (250 mg) was added to the amniotic fluid. After closing the uterus with a running suture in 2 layers using 2-0 vicryl, the uterus was replaced to its normal position and the maternal abdominal wall and skin were closed using 1-0 vicryl. Natrium penicillium (1,000,000 IU) was administered intra-abdominally and depomycin (20 mg/kg, subcutaneously) was initiated preoperatively and maintained postoperatively for 3 days. Fetal viability was assessed by ultrasound postoperatively. The animals were returned to the farm after 3-5 days at the Central Animal Laboratory of the University of Nijmegen.

The second operation was performed 2 weeks later, at 86 or 93 days' gestation. Under general anesthesia a laparotomy was performed as described above. In group 1, the surgically created spina bifida was covered with the defined collagen biomatrix. One horn of the uterus was exteriorized and the fetus was manually located through the uterine wall. A hysterotomy was per-

formed and the spina bifida was exposed. The defect was covered with the collagen biomatrix, sutured to the skin using eight 5-0 prolene interrupted sutures. The fetus was returned to the uterus and amniotic fluid volume was restored with warm saline solution. The uterus, abdominal wall and skin were closed and antibiotics were given as described above. In groups 2 and 3, only an endoscopy without surgical intervention was performed to ascertain wound healing. After positioning of the fetal lamb, a 2-0 vicryl purse-string suture was inserted, which incorporated both the uterine wall and amniotic membranes. A 2-cm incision was made in the uterine wall and the amniotic cavity was opened. A 10-mm balloon cannula (Tyco, The Netherlands) was introduced into the amniotic cavity. The balloon was inflated and the cannula was secured inside the cavity by tying the purse-string suture to prevent leakage of amniotic fluid or carbon dioxide. Carbon dioxide was insufflated at a rate adjusted to maintain the ambient intrauterine pressure. The excess of amniotic fluid was sucked up through the cannula and collected in a sterile bag. A standard 10-mm laparoscope (Karl Storz) was introduced, and the spina bifida or skin lesions were inspected. After fetal inspection, the laparoscope and cannula were removed and the opening was closed with the purse-string suture and a 2-0 vicryl running suture in 2 layers. Prior to closure, the collected amniotic fluid was replaced and the total volume was restored with warm sterile saline solution. The uterus was replaced to its normal position and both the abdominal wall and skin were closed using 1-0 vicryl. Antibiotics were given as described above. Fetal viability was assessed by ultrasound postoperatively. The animals were returned to the farm after 3-5 days at the Central Animal Laboratory.

Postpartum Evaluation

Cesarean delivery was performed under local anesthesia with 20-30 ml 2% lidocaine at 140 days' gestation. The lambs were observed for 1 week and a neurological examination was carried out on day 7 or 8 with the use of a standardized protocol including spontaneous and elicited behavior, with special emphasis on motor, sensory and reflex modalities of hind- and forelimbs, anal sphincter and urinary bladder. Afterwards, the lambs were sacrificed by an intramuscular injection of 10 mg xylazine followed by intracardial injection of 60 mg/kg pentobarbital. In groups 1 and 2, the skull and cervical vertebral column were opened and examined for the presence of hydrocephalus or hindbrain herniation. The vertebral column, spinal cord and brain were excised and fixated in 4% formaldehyde. Cross-sections of the spinal canal and cord with the surrounding tissues were taken at the lumbosacral level,

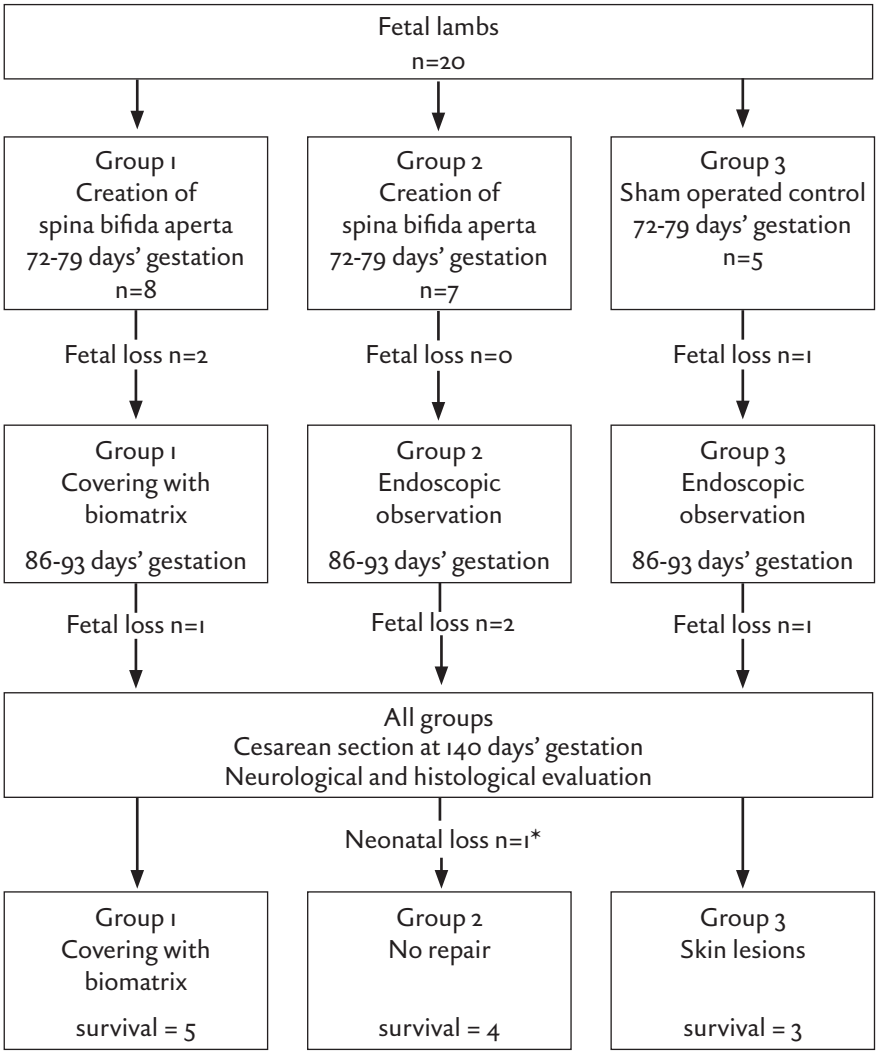
including the site of surgery. In group 3, histological sections of the skin and subcutaneous tissues were taken from the surgically created skin defects. The slides were stained with hematoxylin/eosin and luxol fastblue/hematoxylin. Immunohistochemical staining was performed on paraffin-embedded skin. Sections of 4 μm were incubated with a rabbit polyclonal antibody directed against human collagen I (Monosan, Uden, The Netherlands). As second antibody we used a biotinylated antibody, followed by incubation with an avidin-biotin peroxidase complex (Vector, Burlingame, Calif., USA). Detection was carried out with the use of AEC as substrate (Scytek, Utah, USA). They were studied by light microscopy by a single experienced neuropathologist.

Results

Twenty fetal lambs underwent surgery. Overall, 3 lambs died after the first operation, 4 lambs died after the second operation and 1 lamb died in the neonatal period. The overall fetal survival rate was 65%. Procedures and outcomes for each group are summarized in figure 1. The neurological and histological results are listed in table 1.

In group 1 (spina bifida covered with collagen biomatrix), surgery was performed in 8 fetal lambs. Two lambs died after the first operation. In both procedures, a hemorrhage of the paraspinal soft tissue of the fetal lamb had occurred. One lamb died after the second procedure, 6 days prior to the cesarean section. All second operations were uneventful. Five lambs survived and were available for further evaluation. The skin was closed in 4 of the 5 lambs. In 3 lambs, remnants of the biomatrix were still present above the skin. In one of these lambs a small defect was present; this lamb showed no loss of spinal function but abnormalities of the spinal cord were found on histological examination. The architecture of the spinal cord was disturbed and complex syringomyelia was found. Glial fibers grew from the spinal cord into the dermal scar. The other 4 lambs showed no loss of spinal function and the architecture of the spinal cord was preserved. Syringomyelia was found in 1 lamb and locally, scar tissue merged with the dura and very locally, with arachnoidea. In 1 lamb hydromyelia and a purulent inflammation originating in scar tissue and growing in subdural fat was found. Locally, the inflammation grew into the dura. Locally in 2 lambs, the scar tissue intermingled with the dura. In 1 lamb, minimal damage of the dorsal horn on one level was found. In lambs, with the biomatrix covered with skin, the biomatrix was largely replaced by native collagen and ingrowth of fibroblasts from the surrounding tissues was

FIG. 1. SCHEDULE OF EXPERIMENTS AND SURVIVAL



* Still available for histological evaluation.

found. Small remnants of the biomatrix were found on histological examination (fig. 2). At the level of the spinal cord in this picture, dural and epipial layers are intact. However at a higher level, iron-holding macrophages are found in the dura and the dura itself shows signs of thickening and scarring.
In group 2 (spina bifida uncovered), surgery was performed in 7 lambs. Three operations were complicated by the occurrence of a minor hemorrhage of

the paraspinal soft tissue of the fetal lamb while creating the defect. All lambs survived the first operation and 2 lambs died after the second operation. In one of these lambs it was difficult to visualize the lesion at endoscopy. One lamb died on the 5th day of life and a neurological examination could not be performed but incontinence of urine, indicating impaired neurological function, was found during the observation period. Histological evaluation was performed in all 5 live-born lambs. The skin was closed in all 4 surviving lambs. In the lamb, that died postnatally, the skin was not closed and a small cele (1.7 x 1.2 cm) was present. Within this group, 1 lamb showed loss of spinal function. On histological evaluation, all 5 lambs showed abnormalities of the spinal cord. In 3 lambs the spinal cord had separated (fig. 3), one lamb showed syringomyelia and 1 lamb asymmetry of the spinal cord. In groups 1 and 2 there were no signs of hydrocephalus or hindbrain herniation.
In group 3 (sham-operated controls), surgery was performed in 5 fetal lambs. One lamb died after the first operation and was found dead at endoscopy. In one case, we experienced difficulty performing endoscopy because of the poor visibility. The lamb died after the second operation and was stillborn at the cesarean section. Histology was not performed in these 2 lambs. In the remaining 3 lambs, the operations were uneventful. The uncovered skin lesion on the left side of the back was found closed in all 3 surviving lambs. The covered skin lesion on the right side of the back was also found closed in 1 lamb (fig. 4) while in the other 2 lambs a small defect of the skin was still present. In one of these lambs, remnants of the biomatrix were still visible within the defect. In 1 lamb an inclusion cyst was found on both sides. The cyst on the right side (covered skin lesion) was accompanied with some acute infection and on the left side (uncovered skin lesion) with a deep infiltrating fibrotic inflammation.

Discussion

Seven lambs died during the pregnancy period. In 2 of them, a severe hemorrhage of the paraspinal soft tissue of the fetal lamb occurred while creating the defect, which may explain the death of these lambs. Because of the small amount of total circulating blood volume at this gestational age, every severe hemorrhage can be fatal. The same applies for the subcutaneous hemorrhages, which can be easily caused by frequent manipulations of the fetus. This may be the explanation why 2 lambs died after a difficult endoscopic procedure.

In a previous study, we demonstrated that biomatrices are useful for experimental fetal surgery and, in accordance with other animal studies, acute intrauterine covering of an experimental spina bifida aperta can lead to a better neurological outcome [23,24]. For a better simulation of the natural course of a spina bifida aperta, in utero repair of the experimental defect in this study was delayed for 2 weeks. This study confirmed that covering of an experimental spina bifida aperta preserves the architecture of the spinal cord. A major difference in neurological outcome between the 2 groups could not be demonstrated because also the lambs with the defect uncovered did not demonstrate major neurological complications despite the severely disturbed architecture of the spinal cord. In all 4 surviving lambs of the uncovered group the skin was found closed at birth, so one may speculate that the length of exposure of the spinal cord to the amniotic fluid may have been too short to cause loss of spinal function despite being long enough to cause histological disturbances. Despite the large size of 3 cm of the created defect, spontaneous closure of the skin occurs, which can be attributed to the known excellent healing potential of fetal tissues at this gestational age. It emphasizes that the neurological impairment in the case of a spina bifida aperta is not only caused by the secondary damage of the intrauterine environment to the spinal cord. In the group with the spina bifida covered, 2 weeks of exposure of the unprotected spinal cord to the amniotic fluid did not seem to cause serious damage to the spinal cord. This could be due to the short period of exposure of 2 weeks or to the early gestational age in which the amniotic fluid may not yet be toxic enough to cause damage [25]. A 2-weeks exposure of the spinal cord to the intrauterine environment is clearly not enough to mimic the severity of spinal cord damage in human spina bifida aperta. One lamb of this group showed disturbance of the architecture of the spinal cord, which could possibly be ascribed to the small defect in the skin that was still present at birth. When fibroblasts are bound to an extracellular matrix, such as the collagen biomatrix, the production of new collagen is enhanced. In the process of wound healing, the skin covers the biomatrix. The biomatrix is incorporated in the subcutaneous tissues and degraded by the fibroblast-produced collagenases and replaced by native collagen [26]. This biodegradability of the collagen biomatrix makes it potentially useful for experimental fetal surgery, especially in these cases where primary closure with skin can be difficult. If the biomatrix was replaced by native tissue, it would not have to be removed after birth. Postnatal repair would still be necessary but may be postponed beyond the neonatal period. For use in human fetal repair, the biodegradability of the biomatrix can be changed by alteration of the strength of cross-

linking, the amount of collagen in the suspension or the pore diameter of the matrix.

Endoscopy in this study was performed to gain experience with the procedure in this model and to study whether intrauterine endoscopic coverage of a spina bifida aperta is possible. For this reason, a 10-mm laparoscope was used instead of a smaller instrument. From the difficulties that we encountered, we observed that the sheep model is not suitable to study the endoscopic coverage of a spina bifida aperta.

In conclusion, a collagen biomatrix can be used for in utero coverage of a surgically created spina bifida aperta. When a surgically created spina bifida aperta is covered with a biomatrix, the architecture of the spinal cord is preserved. When this defect is left uncovered, the architecture is disturbed. However, coverage of the defect did not result in a major improvement in the neurological outcome as neurological outcome was surprisingly good despite major disturbance of the spinal cord architecture.

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CHAPTER 5

Intrauterine tissue engineering of full-thickness skin defects in a fetal sheep model

authors
A.J. Eggink*
N.A. Hosper*
L.A.J. Roelofs
R.M.H. Wijnen
M.J.A. van Luyn
R.A. Bank
M.C. Harmsen
P.J. Geutjes
W.F. Daamen
T.H. van Kuppevelt
D.M. Tiemessen
E. Oosterwijk
A.J. Crevels
W.A.M. Blokx
F.K. Lotgering
P.P. van den Berg
W.F.J. Feitz

* These authors contributed equally to this study

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Abstract

In spina bifida the neural tube fails to close during the embryonic period and it is thought that prolonged exposure of the unprotected spinal cord to the amniotic fluid during pregnancy causes additional neural damage. Intrauterine repair might protect the neural tissue from exposure to amniotic fluid and might reduce additional neural damage. Biodegradable collagen scaffolds may be useful in case of fetal therapy for spina bifida, but biochemical properties need to be studied. The aim of this study was to investigate whether biodegradable collagen scaffolds can be used to treat full-thickness fetal skin defects. We hypothesized that the pro-angiogenic growth factors VEGF and FGF2 would enhance vascularization, epidermialization and lead to improved wound healing. To investigate the effect of these two growth factors, a fetal sheep model for skin defects was used. Compared to wounds treated with bare collagen scaffolds, wounds treated with growth factor-loaded scaffolds showed excessive formation of capillaries and less myofibroblasts were present in these wounds, leading to less contraction. This study has demonstrated that collagen scaffolds can be used to treat fetal skin defects and that the combination of collagen scaffolds with VEGF and FGF2 had a beneficial effect on wound healing.

Introduction

There are different congenital structural anomalies in which closure of skin and underlying structures does not occur during embryonic development. In spina bifida (incidence rate 1 of every 2,000 live births) the neural tube fails to close during the embryonic period and it is thought that exposure of the neural tube to the amniotic fluid during pregnancy causes additional neural damage ('two-hit' hypothesis) [1]. Current treatment of spina bifida consists of surgical closure of the defect in the early postnatal period, in order to prevent infection and further damage. However, the damage caused by the exposure to the amniotic fluid is irreversible.

It is expected that intrauterine repair of the defect diminishes the damage caused by exposure to amniotic fluid. An additional advantage of such a fetal intervention is that fetal tissue can regenerate, instead of repair with fibrosis [2]. During the first and second trimester of development, fetal skin wounds heal with a normal epidermal and dermal architecture. During the third trimester a transition from scarless repair to fibrotic repair occurs, which is comparable to adult wound healing with more inflammation and fibrosis [2]. However, the ability of the fetus to heal full-thickness wounds without fibrosis depends on the size of the defect, independent of the gestational age [3].

The aim of this study was to investigate whether biodegradable collagen scaffolds can be used to treat full-thickness fetal skin defects to allow *in situ* fetal dermal repair and epidermal overgrowth. For this purpose we developed a fetal skin defect model in sheep, and treated the defects with a cross-linked type I collagen scaffold (which was previously applied in a rat model and sheep model [4-7]). Collagen scaffolds can be modulated in different ways to enhance and regulate wound healing. The porous matrix structure can be varied and growth factors can be incorporated to promote angiogenesis and cellular ingrowth. In this study we used a collagen scaffold and a collagen scaffold loaded with heparin and two growth factors: vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF2). Heparin was used for optimal growth factor binding. VEGF is a 40-45-kDa homodimeric glycoprotein promoting angiogenesis [8]. FGF2 can also act as angiogenic factor

by directing endothelial cell migration and proliferation. The combination of VEGF and FGF2 has been reported to have potent synergistic effects on neovascularization both in *in vitro* and *in vivo* experiments [9-12]. Furthermore, FGF2 reduces scar formation when exogenously applied to the wound site [13] and diminishes wound contraction, when used in combination with a collagen matrix [14]. FGF2 can also act as a potent mitogen for fibroblasts and keratinocytes [15,16], indicating an effect on both dermal as well as epidermal healing. With the stimulation of vascular ingrowth and cellular infiltration, regeneration of tissue can be improved by supplying sufficient oxygen and nutrients. By regulating the host response to the scaffold in this way, formation of excess scar formation may be prevented.
We evaluated fetal wound healing and closure of full-thickness skin wounds by means of intrauterine tissue engineering using type I collagen scaffolds (COL) and heparinised type I collagen scaffolds loaded with VEGF and FGF2 (COL-HEP/VF), and compared these with untreated defects 2, 4 and 8 weeks after fetal surgery. Cellular ingrowth, vascularization, scar tissue formation and degradation of the scaffolds were evaluated by histology and immunohistochemistry.

Materials and methods

The study has been approved by the local Ethics Committee on Animal Research of the Radboud University Nijmegen, the Netherlands (RU-DEC 2007-239).

Preparation of collagen scaffolds

Type I collagen was purified from bovine Achilles tendon as described previously [17,18]. To prepare collagen scaffolds, a 0.67% (w/v) type I collagen suspension in 0.25 *M* acetic acid was shaken overnight at 4°C and homogenized on ice using a Potter-Elvehjem homogenizer (Louwers Glass and Ceramic Technologies, Hapert, The Netherlands). Air bubbles were removed by centrifugation at 100g for 15 min at 4°C. The suspension was then poured into 6-well plates (4 ml per well), frozen at -20°C, and lyophilized. Scaffolds were pre-incubated with 50 m*M* 2-morpholinoethane sulfonic acid (MES) pH 5.0 containing 40% ethanol for 30 min at 22°C. After removal of this solution, scaffolds were cross-linked using 33 m*M* 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and 6 m*M* N-hydroxysuccinimide (NHS) in 50 m*M* MES pH 5.0 containing 40% ethanol for 4 h at 22°C in the presence

or absence of 0.25% heparin (Diosynth, Oss, The Netherlands). Scaffolds were then washed, frozen and lyophilized. Scaffolds were disinfected in 70% ethanol (6 x 7 ml per well) followed by washings with sterile PBS (8 x 7 ml per well). The cross-linked type I collagen scaffold is abbreviated in the text as COL.

The growth factors vascular endothelial growth factor 165 (VEGF; human recombinant, R&D Systems, Minneapolis, MN, USA) and basic fibroblast growth factor 2 (FGF2; human recombinant, R&D Systems) were loaded onto the heparin-crosslinked collagen scaffolds by incubating six Ø 12 mm scaffolds in 5 ml PBS containing 3.5 μg/ml VEGF and 3.5 μg/ml FGF2, followed by 3 washings with the same volume of PBS. This scaffold is abbreviated in the text as COL-HEP/VF.

Analysis of collagen scaffolds

The ultrastructure of the scaffolds was visualized by scanning electron microscopy (SEM). Specimens were mounted on stubs, sputtered with an ultrathin layer of gold in a Polaron E5100 coating system and visualized with a JEOL JSM-6310 SEM apparatus operating at 15 kV. The degree of cross-linking of the scaffolds was determined spectrophotometrically by determining the amine group content using 2,4,6-trinitrobenzene sulfonic acid. Cross-linking efficiency was expressed as the percentage of the total number of amine groups that were used in the crosslinking process [19,20].

The heparin content of the films was determined applying a hexosamine assay using *p*-dimethylamino-benzaldehyde, taking heparin as a standard [21,22].

The amount of growth factors bound to the scaffolds was determined with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% (w/v) gel, followed by silver staining using a 0.1% (w/v) $AgNO_3$ solution. COL-HEP scaffolds incubated with growth factors and a standard curve of 0-100 ng of either VEGF of FGF2 were incubated for 15 min in a boiling water bath under reducing conditions using 5% (v/v) 2-mercaptoethanol. The intensity of the bands was analyzed using Adobe Photoshop.

The location of growth factors in the scaffolds was visualized by immunohistochemistry using the ABC method [23] with antibodies HS4C3 [24], goat anti-human VEGF (Sigma Chemical Co., St Louis, MO, USA) and rabbit anti-bovine FGF2 (R&D Systems, Minneapolis, MN, USA).

Animals and surgical procedures

Nine pregnant ewes of Dutch Texel breed underwent surgery at 79 days' gestation (term, 140-147 days). For sedation, the animals were pre-medicated with an intramuscular injection of midazolam (0.5 mg/kg). Anesthesia for surgery was induced by an intravenous injection of propofol (5 mg/kg) followed by tracheal intubation. The ewes were maintained on general anesthesia with 1.5% isoflurane. For analgesia, flunixin (2 mg/kg) and sufentanil (4 μg/kg) were given intravenously followed by a maintenance dosage sufentanil of 2 μg/kg/h. Heart rate, temperature, oxygen saturation and carbon dioxide concentration of the expired air were monitored. Fetal anesthesia was achieved by transplacental passage of the medications, administered to the ewe. The abdomen was shaved, cleaned and aseptically prepared. After a low midline laparotomy under sterile conditions, the uterus was inspected for the number of fetuses. One uterus horn was exteriorized, wrapped in gauzes soaked with warm phosphate buffered saline (PBS), and the position of the fetal lamb was determined by palpation. At a favorable location over the caudal side of the fetus a hysterotomy was made.

Three circular skin lesions, 12 mm in diameter, were made on the back of the fetus by excising the skin. The lesion on the left side was covered with a collagen scaffold (COL). The lesion on the right side was covered with the collagen-heparin scaffold with VEGF and FGF2 (COL-HEP/VF). The applied scaffolds were slightly thicker than the removed skin. The lesion in the middle of the back remained uncovered. The scaffolds were secured and marked with four 6-0 prolene (Ethicon, Somerville, NJ, USA) interrupted sutures around the skin lesion. After surgery the fetus was replaced in the uterus and the amniotic fluid volume was restored with warm PBS solution. Amoxicillin (250 mg) was added to the amniotic fluid. The uterus was closed with a running suture in 2 layers using 2-0 vicryl (Ethicon, Somerville, NJ, USA). In case of twin pregnancy the same procedure was performed using the techniques as described above. Sodium penicillin (1,000,000 IU) was administered to the intra-abdominal space and the abdominal wall was closed in 3 layers using EP6 Serafil for the fascia, 2-0 Vicryl for the subcutaneous tissue and 1-0 Vicryl Plus for the skin. Depomycin (20,000 IU penicillin ml^{-1}, 200 mg streptomycin ml^{-1}) was initiated preoperatively (0.6 ml/10 kg) and maintained postoperatively (1 ml/10 kg) for 3 days via intramuscular injections. Buprenorphine (10 μg/kg, intravenous) and flunixin (2 mg/kg, intramuscular) were given for three days to provide postoperative analgesia. The ewes returned to the farm soon after they were able to stand.

Macroscopic evaluation

The animals were sacrificed at three time points. Evaluation took place at 93 days' gestation (2 weeks post surgery), 107 days' gestation (4 weeks post surgery) and 140 days' gestation (term) in group 1, 2 and 3 respectively. Ewes and fetal lambs were euthanized by 10 ml intravenously and 5 ml intracardiacally injected T61® (200 mg embutramide ml^{-1}, 50 mg mebezoniumjodide ml^{-1} and 5 mg tetracainhydrochloride ml^{-1}; Intervet, Boxmeer, The Netherlands).

After macroscopic evaluation and photography of the wound, the size of the defect was measured between the marking sutures and was used to calculate wound contraction. Wound contraction was expressed as the percentage of the size of the defect at time of evaluation against the original size of the defect of 12 mm at time of surgery. All data are represented as mean ± standard error of the mean. The data were analyzed using computer statistical software (GraphPad Prism, GraphPad Software Inc.). The statistic significance of differences in the findings was evaluated by a one-way ANOVA followed by post hoc Bonferroni analysis. A difference was considered statistically significant when $P < 0.05$.

Histological techniques

Samples of the skin and subcutaneous tissue were taken at the level of the skin lesions, fixed in 4% (v/v) buffered formaldehyde and paraffin-embedded for histological analysis or snap-frozen and stored at -80°C. Serial cross sections were cut (4 μm) and stained with haematoxylin and eosin, toluidine blue and Masson's trichrome. Additionally, immunohistochemical stainings for α-smooth muscle actin (α-SMA), pancytokeratin and type IV collagen were performed on paraffin-embedded sections. The sections were deparaffinized and washed with PBS. Endogenous peroxidases were blocked with 3% (v/v) H_2O_2/PBS for 30 min at room temperature. Heat mediated antigen retrieval in Sodium Citrate Buffer (10 mM Sodium Citrate, pH 6.0) during 10 minutes was performed for sections stained for α-SMA and cytokeratin. Sections stained with anti-type IV collagen were treated with 0.1% (v/v) protease (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature for antigen retrieval. Slides were pre-incubated with 5% serum from the species that produced the secondary antibody.

α-SMA was detected with mouse α-SMA (1:15.000, clone 1A4; Sigma-Aldrich) and peroxidase-conjugated goat-anti-mouse antibody (1:200; SBA, Birmingham, USA, cat.no.1080-05). Immunostaining of cytokeratin was performed by overnight incubation at 4°C of mouse anti-cytokeratin (1:800, clone AE1/AE3; Labvision corporation, Fremont, USA), followed by bioti-

nylated horse-anti-mouse and peroxidase conjugated ABC complex (Vector Laboratories, Burlingame, USA). Type IV collagen was detected using rabbit anti-type IV collagen (1:100; Abcam, Cambridge, UK), biotinylated goat-anti-rabbit (Dako) and peroxidase conjugated streptavidine (Dako). Color development was performed with power DAB (α-SMA and cytokeratin, Immunologic, Duiven, The Netherlands,) and 3-amino-9-ethylcarbazole (type IV collagen, Sigma, Steinheim, Germany). Sections were counterstained with Mayer's haematoxylin (Fluka Chemie, Buchs, Switzerland).

Results

Scaffolds

Scanning electron microscopy revealed a porous scaffold with a rather open top side and a more closed pan side (Fig. 1A). Crosslinking efficiency was 31 ± 8% for COL scaffolds and 31 ± 4% for heparinised COL scaffolds (mean ± SD). To heparinised COL scaffolds, 13.1 ± 2.4% heparin was bound (mean ± SD). Heparin was bound evenly throughout the scaffold. The amount of growth factors bound per mg heparinised COL scaffold was 0.26 ± 0.05 μg for VEGF and 0.10 ± 0.04 μg (mean ± SD) for FGF2. FGF2 was present throughout the scaffold whereas VEGF was mainly present at the edges of the scaffold (Fig. 1B).

Animal surgery

Eight ewes and 13 fetal lambs underwent surgery (6 ewes were bearing twins). The overall fetal survival rate was 85% during the pregnancy period. In one sheep, twin pregnancy could not be determined at time of surgery and only one fetus was operated. One sheep aborted 2 days after the surgery probably due to severe enteritis of the ewe and needed to be sacrificed accounting for the only maternal death in the study. At autopsy, herniation of a bowel loop between the fascial sutures was found. In one sheep, a thermal intestinal lesion occurred during the surgery which was repaired. All other procedures were uneventful. Eleven lambs were evaluated; four in group 1 (93 days), three in group 2 (107 days) and four in group 3 (140 days). The survival rate was similar to previously published data for fetal surgery on similarly aged fetal lambs [5-7].

Macroscopic evaluation

A macroscopic overview with examples of wound healing is depicted in Fig. 2A. On macroscopic inspection, the skin defects were visible in all groups.

The untreated defects were completely closed at 8 weeks post surgery, with the wound edges contracted to each other. COL treated wounds showed remnants of the scaffold at 2 and 4 weeks post surgery, at term (8 weeks post surgery) the wound was completely closed without visible remnants of the scaffold. A reddish appearance of the remnants of the COL-HEP/VF treated wounds was present at 2 weeks, suggesting angiogenesis. In contrast to COL treated wounds, COL-HEP/VF treated wounds showed remnants of the scaffold at term.

The percentage of wound contraction of the treated and untreated defects is shown in Fig. 2B. At 2 weeks, the percentage of wound contraction of the COL treated defects was decreased and this was more obvious when using a COL-HEP/VF scaffold. At 4 and 8 weeks, contraction was also found in the COL treated defects but again the contraction of the COL-HEP/VF scaffold was less (~30%). At term, the degree of wound contraction of the uncovered group was almost 80%.

Microscopic evaluation

Two weeks after surgery (93 days' gestation), the *untreated wounds* were covered by a fibrin clot. The newly formed epidermis fully closed the wound area and separated the granulation tissue from the clot; the epidermal cells had migrated over the interface between the clot and the developing granulation tissue. The newly formed epidermis was, at this time point, less than twice as thick as the normal epidermis distal from the wound area (Fig. 3A); the overall thickness was very regular (Fig. 4A). The granulation tissue consisted of a more open extracellular matrix (ECM) network compared to the normal dermis and was populated by numerous cells (Fig. 3A2). The latter appeared to be mainly myofibroblasts as based on their morphology and confirmed by α-SMA staining (Fig. 5A). Compared to the normal fetal dermis in development, less mature blood vessels and capillaries were present in the wounded area (Fig. 3A).

The wounds treated with the COL scaffold showed a fibrin clot covering the top of the scaffold (not shown). Interestingly, the epidermis grew from both sides through the upper part of the scaffold, but did not completely close at this time point. The thickness and shape of the newly formed epidermis was rather irregular (Fig. 3B, 4B) and was generally thicker than the epidermis of the untreated wound (i.e. the wound without a scaffold). New ECM formation occurred at the interface between the scaffold and the epidermis (Fig. 3B1), whereas less ECM formation was observed at the dermal side of the scaffold (Fig. 3B2). Infiltration of cells, mainly α-SMA positive (Fig. 5B),

was seen in the scaffold. In the scaffold only a few small blood vessels were formed (Fig. 3B).

The wounds treated with the COL-HEP/VF scaffold also showed a fibrinous clot on top of the scaffold. Here again, the epidermis grew from both sides through the upper part of the scaffold. However, the newly formed epidermis was much more hyperplastic (Fig. 3C versus B; Fig. 4C versus B) with a highly irregular shape. The wound area was also not completely covered by the epidermis. Less ECM formation was seen at the interface between the scaffold and the epidermis compared to the wound treated with the bare COL implant (Fig. 3C1 versus B1; Fig. 3C2 versus B2) and fewer α-SMA-positive cells infiltrated into the scaffold (Fig. 5C versus B). A few small blood vessels were present in the scaffold (Fig. 3C), which was comparable to the amount in the COL treated wound at this time point.

Four weeks after surgery (107 days' gestation), the clot of the *untreated wounds* was not longer present and the thickness of the newly formed epidermis was completely normalized (Figs. 3D and 4D). A more dense ECM network was formed. A general decrease in cell number was seen (Fig. 3D1 and D2 versus A2); myofibroblasts were only sporadically seen (Fig. 5D). At this time point more small as well as mature blood vessels were observed at the dermal side of the newly formed tissue (Fig. 3D2) compared to two weeks, however less blood vessels than in the normal fetal dermis.

In the wounds treated with COL, the clot was still present (Fig. 3E). The thickness of the newly formed epidermal layer was not normalized (Fig. 4E), but the wound area was completely covered by the epidermis at this time point. Single cells were present between the collagen bundles of the entire scaffold (Fig. 3E2) and numbers of α-SMA-positive cells were even decreased compared to the earlier time point (Fig. 5E versus B). Most importantly, a newly formed ECM layer was present between the epidermis (Fig. 3E and E1) and the scaffold which was devoid of myofibroblasts (Fig. 5E) and in which several blood vessels were formed, comparable to the appearance of the normal dermis. Only a few mature blood vessels were formed in the scaffold as revealed by a collagen IV staining (Fig. 6A).

In the wounds treated with COL-HEP/VF the clot was also still present. The newly formed epidermis was still hypertrophic with an irregular shape (Fig. 4F). ECM formation was mostly observed at the epidermal side of the scaffold (Fig. 3F1) and contained a decreased amount of myofibroblasts (Fig. 5F) compared to the earlier time point. In the scaffold further distal of the epidermis hardly any ECM formation was seen (Fig. 3F2). Compared to the bare COL implant, less cells, mainly α-SMA-negative (Fig. 5F), were present in

the scaffold (Fig. 3F2 versus E2), but a similar amount of blood vessels was present in the scaffold (Fig. 6B versus Fig. 6A).
Eight weeks after surgery (140 days' gestation), the *untreated wound* had completely lost its clot. The granulation tissue was entirely replaced by ECM and had a density comparable with the normal dermis (Fig. 3G). There were no skin appendages in the newly formed tissue (Fig. 4G) and myofibroblasts were absent (Fig. 5G). A comparable amount of mature blood vessels was present in this newly formed ECM (Fig. 3G) when compared to the normal fetal dermis.
In the wounds treated with COL, the clot was also lost, whereas still parts of the epidermis were thickened (Fig. 3H). Interestingly, the entire scaffold was degraded and replaced by ECM (Fig. 3H). A decreased amount of myofibroblasts was seen compared to four weeks (Fig. 5E versus H). A higher amount of blood vessels was present throughout the newly formed ECM layer (Fig. 3H1, H2 and 6C) compared to the normal dermis.
In contrast, in the wounds treated with COL-HEP/VF part of the clot was still present. The thickness of the epidermal layer had decreased (Fig. 3I and 4I), but was still thicker than the newly formed epidermis of the untreated and the COL treated wound. Remarkably, some of the skin appendages of the epidermis were formed in the newly formed ECM (Fig. 3I1 and 4I). This ECM layer was not as dense as the newly formed ECM in the untreated and the COL treated wound. In contrast to the latter, a considerable part of the scaffold was still present (Fig. 3I). Although it still concerned low numbers, a higher influx of cells was seen in the scaffold at this time point compared to four weeks after treatment (Fig. 3I2 versus F2). The amount of myofibroblasts was decreased compared to the four week time point (Fig. 5I versus F). Besides mature blood vessels, many capillaries were formed in the scaffold (Fig. 6D and DI).

Discussion

Fetal tissue engineering has been proposed as a new concept in the surgical reconstruction of birth defects in the fetal period [25]. However, despite being a promising concept, hardly any studies have been published to tackle the various steps needed to establish a proof-of-concept. In this study we investigated whether biodegradable scaffolds with or without growth factors can be used to treat full-thickness fetal skin defects which facilitate epidermal overgrowth and dermal repair. We studied the healing of these skin

defects by use of a bare collagen type I (COL) scaffold, a heparinised COL scaffold loaded with VEGF and FGF2 (COL-HEP/VF), or left untreated (no scaffold). We hypothesized that the pro-angiogenic growth factors VEGF and FGF2 might enhance vascularization and epithelialization, leading to improved wound healing.

The study showed that at term (8 weeks post surgery) proper epidermal layers were formed in all defects. Less wound contraction occurred in COL treated defects compared to untreated defects. Even less contraction was present in defects treated with COL-HEP/VF, but in contrast to COL treated defects, degradation of the scaffold was not completed at term. Concerning degradation of the scaffold, hardly or no macrophages infiltrated the COL and COL-HEP/VF scaffolds, suggesting that these cells did not play a role in fetal tissue engineering. Similar amounts or even less blood vessels were formed in the defects compared to the developing fetal dermis, with exception of COL-HEP/VF treated defects at term, showing excessive formation of capillaries in the scaffold. Overall, myofibroblasts were involved in the remodeling of the tissue, i.e. ECM formation and wound contraction.

It is highly important that wound contraction and scar formation will be minimized after fetal skin defects, as the fetus grows rapidly. Therefore we investigated the formation of ECM and the amount of contraction in time. Previously, a fetal sheep model has shown that the transition period from scarless to healing with scar formation in sheep occurs between 100-120 days [3]. However, fetal skin repair is not only dependant on gestational age, but also on the size of the defect. Wounds in mid-gestation fetal lambs of 2-4 mm heal without scarring, whereas wounds of 6-10 mm heal with formation of a scar [3]. In our study, wounds of 12 mm were made at 79 days of gestation. It was expected that the untreated wounds would heal with scar formation due to the size of the wound. Indeed, all untreated defects showed healing with scar formation and contraction. However, clearly less wound contraction was observed with COL treatment. Myofibroblasts have contractile characteristics in an attempt to close the wound. Another function of myofibroblasts is the production of new ECM components. A comparable amount of myofibroblasts was seen at two weeks post surgery in untreated and COL treated wounds. At 4 and 8 weeks myofibroblasts were still present in COL treated wounds, resulting in a larger area of newly deposited ECM. As hypothesized, treatment of the skin defect with COL-HEP/VF diminished contraction even more. In accordance with this, fewer myofibroblasts were present. This is probably due to the incorporation of FGF2, because *in vitro* and *in vivo* studies have shown that FGF2 can inhibit and reverse the differentiation of fibroblasts to myofibroblasts [13,14,26,27].

Implantation of a biomaterial, like our degradable type I collagen scaffold, will induce an inflammatory response by the non-specific immune system, known as the foreign body reaction (FBR). In general the FBR will induce resorption of degradable scaffolds after implantation. Key players in the adult FBR are macrophages and giant cells. However, little is known about the FBR in fetal applications. It is known that in fetal wound healing less inflammation is induced compared to adult wound healing, which can be due to the fact that the innate immune system is less mature in the fetus. In the current fetal lamb model hardly any or no macrophages were detected in any of the histological specimens, and most of the cells were myofibroblasts. We wanted to confirm the scarcity of macrophages by a macrophage staining, but antibodies against sheep macrophages are not available. Several other macrophage antibodies have been tested to see if these cross-react, but none of these antibodies were successful. In accordance with the absence of macrophages, also giant cells (fused macrophages) were not present in the fetal skin wounds. This is a major difference with the adult situation in which implanted biodegradable scaffolds are finally phagocytosed by giant cells. In the current fetal model, COL and COL-HEP/VF scaffolds did degrade but as mentioned giant cells were not involved, suggesting that the scaffolds were degraded by enzymes secreted by (myo)fibroblasts.

In fetal tissue engineering it is important that a good balance exists between the degradation of the scaffold and new tissue formation, i.e. epithelial overgrowth and proper dermal regeneration. We observed a faster tissue remodeling in the COL treated wounds compared to COL-HEP/VF treated defects. At four weeks in COL treated wounds, part of the collagen scaffold was degraded and replaced by an ECM layer that integrated with the original dermis. At eight weeks, the entire scaffold was degraded and replaced by new ECM. In contrast, in the COL-HEP/VF treated wounds, a large part of the scaffold was still present at 8 weeks post surgery, demonstrating that remodeling is still in progress. These differences between COL and COL-HEP/VF implicate that fetal TE can be influenced by growth factors leading to different degradation patterns.

Another major difference between COL and COL-HEP/VF treated defects was the hyperplasia of the epidermis observed at 2 and 4 weeks post surgery in COL-HEP/VF treated wounds. This could be caused by FGF2 as well as VEGF. It is known that FGF2 can have a mitogenic effect on keratinocytes, thereby promoting epithelialization in adult wound healing [15,16]. VEGF can also stimulate the proliferation and migration of keratinocytes *in vitro* [28]. Furthermore, application of VEGF in a diabetic wound healing model

showed accelerated wound healing with enhanced epithelialization [29]. However, finally at 8 weeks a proper epidermal layer has been formed. Furthermore, enhanced angiogenesis was observed in the COL-HEP/VF treated wounds. Both VEGF and FGF2 can promote angiogenesis and the combination of VEGF and FGF2 has been shown to have synergistic effects on neovascularization both *in vitro* and *in vivo* [9-12]. Although excessive formation of capillaries was observed in these scaffolds 8 weeks post surgery, we do not know whether these capillaries are functional, because erythrocytes were not observed in these capillaries.

A highly interesting observation is the formation of new appendages in the wounded area of the COL-HEP/VF treated wounds 8 weeks after surgery. Regeneration of these specialized structures indicates an enhanced functional healing. These newly formed appendages were less mature (smaller compared to appendages in the normal skin at this time of development) than the appendages in the unwounded skin, indicating that the appendages have not migrated from the wound edges.

For optimal binding of growth factors to the scaffold, the scaffolds were heparinised. We have to realize that directly after implantation the scaffold is in contact with the amniotic fluid. It is known that several growth factors are present in the amniotic fluid, like hepatocyte growth factor (HGF), insulin-like growth factor (IGF), FGF2, and VEGF. We can not exclude that, in addition to the loaded growth factors, also growth factors from the wound site or from the amniotic fluid bound to the heparin in COL-HEP/VF, leading to a long-term effect of these growth factors.

For future tissue engineering approaches of congenital defects such as spina bifida, scaffolds could be useful for wound closure. In utero coverage of the defect with a scaffold will shorten the exposure time of the spinal cord to the amniotic fluid, which will likely improve the neurologic outcome by preventing further damage. However, future studies will be needed to investigate if the addition of growth factors is beneficial in the case of spina bifida.

Conclusion

The evaluation of biomaterials for specific applications should be evaluated in model systems resembling the clinical situation. This study showed that tissue engineering in utero is a promising method in treatment of full thickness fetal skin defects. Both COL and COL-HEP/VF treated wounds showed a proper re-epithelialization and healing without a (chronic) FBR. COL treated

wounds show less contraction than untreated wounds and this effect was even enhanced in COL-HEP-VF. Furthermore, the addition of these growth factors leads to an increased angiogenesis and to regeneration of the skin appendages.

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CHAPTER 6

Amniotic fluid exchange in an experimental neural tube defect sheep model: histological outcome and fetal waste concentrations

authors
A.J. Eggink
L.A.J. Roelofs
W.F.J. Feitz
R.M.H. Wijnen
M.M.Y. Lammens
T.H. van Kuppevelt
A.J. Crevels
P.J. Geutjes
F.K. Lotgering
P.P. van den Berg

Submitted

Abstract

Introduction: In myelomeningocele, prolonged exposure of the unprotected spinal cord to harmful substances in the amniotic fluid (AF) has deleterious effects on the neural tissue. The aim of the study was to evaluate the effect of amniotic fluid exchange (AFE) on neural tissue damage in fetal sheep with a surgically created neural tube defect and to measure the concentrations of urinary and gastrointestinal waste products in the AF before and after the procedure.

Material and Methods: At 79 days' gestation, an experimental neural tube defect was created in 11 fetal lambs. In 6 sheep, only samples of the AF were collected at a regular interval for assay of waste products. In 5 sheep, AFE was performed at regular intervals and samples of AF were collected and analyzed.

Results: After 1 week almost all concentrations returned to baseline concentrations prior to AFE and no difference in histological outcome was found between the two groups.

Discussion: Urinary and gastrointestinal waste product concentrations return to normal within 1 week after AFE. AFE with a frequency of 1 week does not reduce neural tissue damage in fetal lambs with surgically created spina bifida aperta and is not useful for removal of waste products.

Introduction

Prolonged exposure to amniotic fluid (AF) has been reported to have a deleterious effect on tissues that normally do not come into contact with AF, e.g. in congenital anomalies in which closure of skin and underlying structures does not occur during embryologic development (e.g. myelomeningocele, gastroschisis and bladder exstrophy) [1-7]. In myelomeningocele, experimental studies have shown that, the absence of the dermal-musculoskeletal cover results in persistent exposure of the unprotected neural tissue to AF which may lead to progressive chemical damage. In utero repair of a myelomeningocele might protect the neural tissue from exposure to AF and arrest the process of progressive neural tissue destruction [8-14].
In myelomeningocele, urinary waste products, in particular, urea have been associated with neurotoxic effects on the exposed neural tissue [15-17]. However, experimental evidence suggests gastrointestinal waste products in the AF rather than urinary waste products may be responsible for the neural damage [18].
AF exchange (AFE) is performed by partial withdrawal of AF, followed by replacement with an equal amount of warm sterile physiologic saline solution to reduce and dilute deleterious waste products.
The aim of the study was to evaluate the effects of AFE on the concentrations of waste products in the AF compartment before and after the exchange procedure and to assess any reduction in histological neural tissue damage.

Material and methods

The study was approved by the Ethical Committee on Animal Research of the Radboud University Nijmegen, The Netherlands (CDL 27018/19).
Eleven sheep of Dutch Texel breed underwent surgery at 79 days' gestation (term, 140-147 days). After induction of general anesthesia with an intravenous administration of pentobarbital (20-25 ml; 60 mg/ml) and atropine (1 ml; 0.5 mg/ml), the ewes underwent tracheal intubation and were maintained on

general anesthesia with 1.5-2% isoflurane in a mixture of oxygen and nitrous oxide. Heart rate, oxygen saturation and concentration in the inspired air and carbon dioxide concentration in expired air were monitored. Fetal anesthesia was achieved by transplacental passage of the medications administered to the ewe. The procedure took place under sterile conditions. The abdominal cavity was opened by low midline laparotomy and one horn of the uterus was exteriorized. The position of the fetal lamb was determined by palpation and a hysterotomy was performed over the caudal side of the fetus. After opening of the uterus, AF was collected in a sterile syringe and the hindlimbs and back of the fetus were exteriorized. Uterus and fetal lamb were wrapped in warm saline-soaked gauzes. An experimental neural tube defect (NTD) was created in the fetal lamb by excision of the skin (diameter 3 cm), paraspinal muscles and soft tissue. Laminectomy was performed of 3 lumbar vertebrae (L_{3-5}). The dura was opened in the midline and the outward flow of liquor was confirmed (fig. 1). The lesion was left uncovered and the fetus was replaced into the uterus. The AF volume was restored with warm saline solution and amoxicillin (250 mg) was added to the AF. Two catheters (diameter 3 mm) were left in the amniotic cavity and fixed to the uterine wall with an interrupted 2-0 vicryl suture (Ethicon, Somerville, NJ, USA). The uterus was closed with a running 2-0 vicryl suture in 2 layers and replaced to its normal position. Both catheters were exteriorized to the lateral abdominal side of the ewe through the abdominal cavity and were lead to the outside through a small opening in the skin. They were fixed to the skin using interrupted 2-0 vicryl sutures and stored in a pouch attached to the flank of the ewe. In the last two sheep (one in each group) a subcutaneous port-a-cath system (Deltec, Inc., St.Paul, USA) was used instead of catheters for sampling and exchange. Sodium penicillin (1,000,000 IU) was administered to the intra-abdominal cavity and the abdominal wall was closed in 2 layers using 1-0 vicryl. Depomycine (20 mg/kg, s.c.) was initiated preoperatively and maintained postoperatively for 5 days. Fetal viability was assessed by ultrasound postoperatively. The animals stayed for a few days at the laboratory and were fed normally before they were returned to the farm.

The animals were divided into two groups. In group 1 (n=6), samples (10 ml) of the AF were collected at a regular interval of 1-3 days (n=3) or 7 days (n=3) for biochemical analysis of urea, creatinine, amylase, alkaline phosphatase, gamma-glutamyltransferase (GGT) and bile acids. AF was placed on ice immediately after collection and then stored at -80°C until analysis. In group 2 (n=5), AFE was performed at regular intervals of 2 days (n=1) or 7 days (n=4) until the moment of birth. A maximum of 400 ml AF was aspirated and replaced

with warm sterile physiologic saline solution (0.9%). Before and after the procedure, samples of the AF were collected at regular intervals, placed on ice and stored at -80°C until analysis. At the end of each procedure, amoxicillin was instilled into the catheter in both groups to prevent infection. With each sample, AF was also collected for microbiological analysis. If necessary, other antibiotics were given depending on the microbiological outcome.
Cesarean section was performed at 140 days' gestation under local anesthesia with lidocaine (20-30 ml; 2%). The lambs were examined macroscopically at birth. The defect was left uncovered. After one week the lambs were sacrificed by intramuscular injection of xylazine (10 mg) followed by intracardial injection of xentobarbital (60 mg/kg). Tissue samples of the spinal cord and column and surrounding tissues were taken at the lumbosacral level including the site of surgery, fixated in 4% (v/v) formaldehyde in 10 m*M* phosphate buffer (pH 7.2) for at least 24 h at 4°C and embedded in paraffin. Consecutive 5 μm sections were mounted onto glass slides, dewaxed in xylol and rehydrated through a graded series of ethanol. Haematoxylin and Eosin (HE), HE-Luxol fast blue and Elastin Van Gieson (EVG) stainings were employed to histologically evaluate each sample with conventional light microscopy by an experienced neuropathologist for changes in the architecture of the spinal cord.

Results

Successful experiments in both groups with evaluable data were achieved in 5 of 11 animals. The other 6 animals were lost to follow up due to intrauterine infection and consequently abortion. The fetal survival rate was 45%. No maternal deaths occurred in both groups. In most cases the microbiological cultures of the aborted lambs (n=7) were positive for Streptococcus, Staphylococcus, Escherichia coli or Candida. In 2 ewes a port-a-cath system was used instead of catheters because of the high infection rate. Six of the 11 surgical procedures were uneventful. In 5 cases a small hemorrhage of the paraspinal soft tissue occurred, but adequate hemostasis could be achieved.
In group 1 (n=6), 3 sheep aborted (postoperative day 18,21 and 35 respectively) and 1 sheep delivered spontaneously at 138 days' gestation. In one sheep the catheters were damaged during transport to the farm and sampling could not take place. Sampling was performed in 5 of 6 lambs. In 3 of 6 lambs histological evaluation was performed.
In group 2 (n=5), 4 sheep aborted (postoperative day 19, 21, 24 and 51 respec-

tively). In one sheep the catheters were damaged during transport to the farm. The volume of AF that could be exchanged varied between 100 and 400 ml. In the 2 lambs with the port-a-cath system, only infusion with saline was performed after 2 exchange procedures because no AF could be withdrawn from the amniotic cavity. Sampling was performed in 4 of 5 lambs. Histological evaluation was performed in 2 of 5 lambs.

The major macroscopic and histological findings are listed in table 1. In summary, all 5 lambs showed major histological abnormalities of the spinal cord at the lumbar level. No differences were seen between both groups. Figure 2 shows an example of the abnormal architecture of the spinal cord in both groups besides a normal spinal cord at different lumbar levels.

Biochemical constituents in the AF of one sheep of group 2, before and after AFE are presented in figure 3. AFE was performed at 4 time points with 350, 300, 180 and 120 ml warm sterile physiologic saline solution (0.9%) respectively. The first exchange was performed 3 days after surgery. Measurements, 1h after exchange show a sharp decrease in concentrations of both urinary and gastrointestinal waste products. After the second and third AFE, it is visible that the concentration of waste products returns to their level before exchange within 2-3 days.

Table 2 represents the mean change of concentration of biochemical constituents in the AF at 93 days' gestation expressed as a percentage of the starting point at 86 days' gestation. Concentrations at 86 and 93 days' gestation were available in 4 of 6 sheeps in group 1 and 2 of 5 sheeps in group 2. These results demonstrate that after 1 week almost all concentrations reached their level of concentration before AFE. Urea and amylase reached a mean concentration of 77 and 67 percent respectively of the starting point concentration at 86 days' gestation. From the data from individual animals we know that urea sometimes reached a level of 93 percent of the starting point concentration 1 day after AFE and this can also be seen in figure 3.

Discussion

Amniotic Fluid Exchange is performed by partial withdrawal of AF followed by replacement with an equal amount of physiologic saline solution. It is supposed to act by reducing concentrations of deleterious waste products in the AF. Olguner et al. [19] showed in a study on chick embryos that neural tissue damage can be prevented by AFE. Correia-Pinto et al. [18] demonstrated that exposure of a surgically created meningomyelocele in fetal rats to meco-

nium leads to severe functional impairment and that necrosis of neural elements was increased in those animals that were exposed to diluted human meconium in the AF. Urea may have cytodestructive or neurotoxic effects on exposed neural tissue but these results support the hypothesis that besides urinary waste products, intestinal waste products may be held responsible for the neural tissue damage in spina bifida aperta [17]. This is also supported by the study of Danzer et al. [20] which demonstrated that meconium passage appears to occur early in fetal life in case of a myelomeningocele. Normally a sharp decrease of digestive enzymes in the amniotic fluid is found after 18 weeks due to maturation of the anal sphincter and accumulation of meconium in the cecum but this may be impaired in some cases with myelomeningocele due to anal incontinence and defective anal innervation leading to continuous in utero defecation, meconium leakage and abnormally high concentrations of digestive enzymes in the amniotic fluid [21].

In different animal models on gastroschisis, AFE proved to be effective in terms of reducing intestinal damage, however a substantially beneficial effect in human studies has not yet been clearly demonstrated [5,22-24]. Only two small human studies demonstrated a positive result of amniotic fluid infusion and AFE in the clinical outcome of children born with gastroschisis but in some of these cases oligohydramnios was also present [25,26]. There is no consensus on the volume and frequency of exchange. The frequency varied between 1 to 3 weeks and the volume between 50 and 500 ml.

This is the first study in which the effect of AFE on neural tissue damage is examined in a larger animal model. Overall we experienced a low survival rate of 45%. Comparing to other studies with survival rates between 65 and 87%, we must conclude that the abortion rate was higher than expected [11,13]. This may be explained by the high infection rate. Despite the use of antibiotics during the AFE, the presence of the catheters made the procedure vulnerable for infections. For this reason, the protocol was changed and in the last two sheep a port-a-cath system was used. A port-a-cath system is implanted subcutaneously where it can be maintained in a comfortable and sterile condition for use in clinical human conditions but also in long term projects in laboratory animals [27]. Because there is no chronic exit site wound, infection risks are considerably lower than with external catheters. In this study we demonstrated that a port-a-cath system can also be used for sampling AF and to administer medications in the amniotic cavity. It turned out to be less suitable for AFE.

No difference in both macroscopical and histological outcome could be observed between the two groups. In both groups the architecture of the

spinal cord was disturbed. Concerning the results of the waste products in AF after AFE and the interval of 1 week between the exchanges, the lack of histological difference is not surprising. Most of the concentrations were normalized after one week or even earlier and this is well illustrated in figure 3. As the study groups are very small, no hard conclusions can be drawn from this results.

Concentrations of urea and amylase reached a mean concentration of 77 and 67 percent respectively of the starting point concentration at 86 days after 1 week. However, threshold levels of intraamniotic concentrations of possible harmful substances to cause neuronal damage are not known.

In the study on chick embryos of Olguner et al. [19] AFE was performed with an interval of 24 hours. After 24 hours we found that most of the gastrointestinal waste products have not reached their initial level so with this interval a substantial dilution of the gastrointestinal waste products in the AF may be realized. However an interval of 24 hours of AFE is not recommendable because of the invasiveness and side effects of the procedure. If, according to other studies, urea is responsible for the neural tissue damage, AFE will not be effective in reducing neural tissue damage because urea reaches the starting point concentration already within 24h in most of the cases. Data of biochemical analysis of the AF support the hypothesis that there is a fast turnover of AF in the third trimester [28,29].

As gestation proceeds, creatinine, urea and alkaline phosphatase show an increase in concentration in the AF towards term according to other studies [30-32]. The increase in creatinine, being more pronounced than that of urea, reflects of course the maturation of tubular function. By contrast, amylase, bile acids and GGT show a decrease in concentration in the AF.

In summary, our results indicate that the frequency of AFE of 1 week is too low to reduce neural tissue damage in fetal lambs with a surgically created spina bifida aperta. The fast turnover of the AF makes that urinary and gastrointestinal waste products, which supposed to be harmful for the unprotected spinal cord tissue, will reach their normal concentration in the AF within 1 week after AFE.

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TABLE 1. MACROSCOPIC AND MICROSCOPIC EVALUATION OF LAMBS WITH A SURGICALLY CREATED NTD WITHOUT AND WITH AFE (L=LUMBAR LEVEL OF THE SPINAL CORD). IN GROUP 1 (NTD WITHOUT AFE, N=6) AND GROUP 2 (NTD WITH AFE, N=5), 3 AND 2 LAMBS WERE AVAILABLE FOR MACROSCOPIC AND MICROSCOPIC EVALUATION RESPECTIVELY

Group	Lamb No.	Macroscopic appearance	Level	Microscopic appearance of the spinal cord
group 1 NTD without AFE	1	no skin defect	L1	posterior funiculi are missing
			L2	posterior funiculus and posterior horns are missing
			L3	posterior funiculi, posterior horn and central canal are missing, cyst formation
			L4	normal
			L5	normal
	2	small skin defect	L1	normal
			L2	posterior funiculi slightly compressed
			L3	posterior funiculi compressed and asymmetrical with cyst formation
			L4	fused dura
			L5	normal
	3	large cele (50x15 mm)	L1	not evaluated
			L2	normal
			L3	fused posterior horn, posterior funiculi partly missing, dilated central canal
			L4	small remnant of the spinal cord fused with the dermis
			L5	split spinal cord with fused posterior funiculi and horns, dura is missing, inflammation in spinal cord and dermis
group 2 NTD with AFE	1	no skin defect	L1	central canal slightly closed
			L2	posterior funiculi compressed and almost absent with cyst formation
			L3	fused dura and abscess formation
			L4	normal
			L5	normal

Group	Lamb No.	Macroscopic appearance	Level	Microscopic appearance of the spinal cord
	2	large cele (20x15 mm)	L1 L2 L3 L4 L5	not evaluated normal dura fused with subcutaneous tissue dilated central canal, hydromyelia, inflammation in dermis cyst formation in flattened anterior horn, syringo- and hydromyelia, dilated central canal, posterior horn fused with subcutaneous tissue

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CHAPTER 7

Reconstruction of congenital birth defects in fetal sheep models: 10 years single-institution experience

authors
A.J. Eggink
P.J. Geutjes
L.A.J. Roelofs
A.J. Crevels
A.E.J. Hanssen
C. van den Broek
W.F. Daamen
T.H. van Kuppevelt
R.M.H. Wijnen
P.P. van den Berg
W.F.J. Feitz

Submitted

Abstract

Introduction: To study the use of tissue engineering techniques for the treatment of congenital birth defects in the pre- and postnatal period, several different sheep models are currently investigated. Several congenital defects were reconstructed in sheep models using collagen-based materials. According to the 3 R's (replacement, refinement and reduction), adaptations to the protocols were made for the re-use (reduction) and refinement to improve animal welfare.

Materials and methods: Pregnant sheep underwent fetal surgery around 79 days' gestation. A neural tube defect, gastroschisis, bladder exstrophy or skin defect was created surgically in the fetal lamb and reconstructed with different highly molecularly defined type I collagen scaffolds in the fetal or neonatal period.

Results: One hundred and seventy two surgical procedures were performed in 163 pregnant sheep divided over 12 different study protocols. The experiment could be finished successfully in 65% of all cases. The overall fetal survival rate was 74% during the pregnancy period. There were 5 maternal deaths due to complications, most of them were due do abdominal wound dehiscence.

Discussion: The sheep model is a well suited large animal model to perform fetal interventions (e.g. to create congenital defects) and to study the use of collagenous scaffolds for the treatment of these severe defects. With refinements of our study protocols, the number of animals used could be reduced and the welfare of the laboratory animal enhanced.

Introduction

Treatment of congenital birth defects is still challenging. Outcomes are still disappointing in extreme difficult cases. Because therapy can have major implications for the quality of life, innovative techniques are developed in this field of healthcare. The use of a biological substitute, designed to maintain, restore or improve tissue function is one of the promising approaches to treat newborns with severe birth defects. Biocompatible and biodegradable scaffolds may serve as a template to guide the growth and organization of wound healing [1].

Different fetal sheep models have been developed and used to investigate tissue engineering techniques and new treatment options for congenital structural anomalies [2-15]. Our research group has used several sheep models to study the reconstructive and regenerative properties of different collagen scaffolds with respect to improvement of the clinical outcome using intra uterine treatment [16-21]. Fetal sheep models for neural tube defect, gastroschisis and bladder exstrophy have been used to study whether tissue engineering techniques can improve the clinical and histological outcome after birth. Intrauterine covering of an experimental neural tube defect with a collagen scaffold can preserve the architecture of the spinal cord which consequently leads to a better outcome [16-18,22]. Also, fetal bladder wall regeneration in bladder exstrophy and fetal abdominal wall tissue regeneration in gastroschisis using a collagen scaffold is feasible in animal models [19,20]. A fetal skin model was used to study the effects of collagen scaffolds with or without heparin and growth factors on the surrounding tissue and the foreign body reaction in a full-thickness skin defect [21]. For these experiments, animal models are indispensable since application in humans is not yet technically or ethically possible.

In the past 10 years, 172 surgical procedures on fetal lambs were performed. Despite the promising results, these studies warrant further description of the biotechnical details on the sheep models. In this paper, we share our experience with the use of fetal sheep models, with respect to survival rate, surgery, biotechnical details, complications, refinements and reductions.

Materials and methods

Approval from the Ethical Committee on Animal Research of the Radboud University Nijmegen Medical Centre (RUNMC), The Netherlands, was obtained for each study separately.

In total, 163 pregnant sheep (172 lambs) of Dutch Texel breed or a crossbred between Swifter breed and Texel breed of known gestational age underwent surgery at 79 days' gestation (term, 140-147 days). The number of included animals per study is listed in table 1.

Fertility protocol. Sponging was performed in 2 ewes simultaneously. On day zero, ewes received intravaginal sponges containing medroxyprogesterone acetate (veramix, 60 mg, Pfizer, Capelle a/d IJssel, The Netherlands) for 12 days followed by injection of 500 IU of pregnant mare serum gonadotropin (PMSG, folligonan, 500-750 IU subcutaneous, Intervet, Boxmeer, The Netherlands) at sponge removal. Since dosage of PMSG is season dependent, 500 IU was given during and 750 IU after regular breeding. A fertile ram was allowed with the ewe on day thirteen, one day after sponge removal. In this way, gestational age was exactly known at the time of operation.

Preoperative management. All collagen-based scaffolds were made by the Biochemistry Department, RUNMC, Nijmegen, The Netherlands. In the last studies, the experiment animal was accompanied during the transport from the farm to the animal laboratory and/or stay by a 'buddy sheep'. Fresh grass and grass hay were withdrawn 48h and 16h before surgery respectively and ewes were weighed. In all studies, except the last, anesthesia was induced by an intravenous injection of pentobarbital (17-45 ml; 60 mg/ml, Faculty of Veterinary Medicine, Utrecht, The Netherlands) and atropine (0.5-1 ml; 0.5 mg/ml, Pharma Chemie, Haarlem, The Netherlands). In the last study, animals were premedicated with an intravenous injection of propofol (5 mg/kg, B. Braun, Melsungen, Germany). Following endotracheal intubation, the ewes were maintained on general anesthesia with 1.5-2% isoflurane (Nicholas Piramal, London, UK) and O_2/N_2O or, after the first study, O_2/air ventilation. In the last study, flunixin (2 mg/kg, Schering Plough, Segre, France) and sufentanil (4 μg/kg, Janssen Cilag BV, Tilburg, The Netherlands) were given intravenously followed by a maintenance dosage sufentanil of 2 μg/kg/h for analgesia. Heart rate, temperature, oxygen saturation and carbon dioxide concentration in the expired air were monitored. Fetal anesthesia was achieved by transplacental passage of the medications, administered to the ewe.

Intra operative management.

Due to the complexity of most procedures, surgery was performed on only one fetus in case of twin or triplet pregnancy except for the skin defect protocol in which surgery was performed on both fetuses in case of twin pregnancy. For all protocols there were 3 surgeons who performed the operations, based on logistics. Concerning all experiments, 5 surgeons and 2 operating room assistants were involved. In all studies, surgery took place under aseptically conditions. The abdominal wall was shaved, washed with soap and aseptically prepared and sterilely draped before a low midline laparotomy was performed (Fig. 1A, B and C). After palpation to determine the number and position of fetuses, the appropriate horn of the uterus was exteriorized and a hysterotomy was performed by opening the uterus diathermically parallel to the vasculature (Fig. 1D and E). Depending on the type of surgery, with minimal handling of the fetus to prevent the formation of subcutaneous hematoma, hindlimbs and back (neural tube defect, skin defect) or the abdominal wall (gastroschisis and bladder exstrophy (Fig. 1F)) was exposed through the uterine incision. The exteriorized uterine horn and fetus were wrapped in gauzes soaked with warm phosphate buffered saline (PBS). According to the type of protocol, the specific surgery was performed as described earlier [16-21]. In the study on neural tube defects, a neural tube defect was created surgically by excising skin and paraspinal muscles and soft tissue and performing a laminectomy of 3 lumbar vertebrae (L_{3-5}) [16-18]. The dura was opened in the midline over the length of 3 vertebrae (Fig. 2A). The lesion was left uncovered, covered with a collagen scaffold or closed by suturing the skin over the defect. In the first study, covering was performed immediately, in the second study, coverage was delayed for two weeks. In the second study, in some groups also an endoscopy in the amniotic cavity was performed during the second procedure. In the third study, a neural tube defect was created, left uncovered and catheters were left in the amniotic cavity to perform amniotic fluid exchange during the remainder of pregnancy. In the gastroschisis study, an incision was made in the left lower quadrant of the fetal abdominal wall [20]. The bowel was exposed through this full-thickness abdominal wall defect and the lesion was left uncovered (Fig. 2C) or the bowel was manipulated back into the abdominal cavity and covered by a collagen scaffold. In the bladder exstrophy study a vertical infra-umbilical median incision was made through the skin in female fetuses and a paramedian skin incision was made next to the urethra in male fetuses followed by a midline incision of the abdominal wall [19]. The bladder was exposed and an incision was made into the anterior bladder wall. In one group the edges of the bladder wall

were sutured to the abdominal wall resulting in an exstrophic bladder (Fig. 2B). Depending on the protocol, the lesion was left uncovered or closed by placing a collagen scaffold into the bladder defect and closing the abdominal wall. Postnatal reconstruction of the bladder defect was performed in an additional protocol. In the skin protocol, three circular lesions were made on the back of the fetus by excising the skin [21]. The lesions were left uncovered or covered with different types of collagen scaffolds (Fig. 2D). Scaffolds were sutured to the surrounding tissues and, if necessary, marked with interrupted sutures (monocryl 6-0 and prolene 6-0, Ethicon, Norderstedt, Germany) [23]. After the surgical procedure, the fetus was replaced into the uterus and the amniotic fluid was restored with warm sterile PBS solution together with amoxicillin (250 mg, Centrafarm Services B.V., Etten-Leur, The Netherlands). The uterus was closed in two layers with a running 2-0 vicryl suture (polyglactin 910, Ethicon, Somerville, NJ, USA). Gestational membranes were closed with the uterine wall in the first layer. Sodium penicillin (1,000,000 IU, Astellas Pharma, Leiderdorp,The Netherlands) was administered into the intra abdominal space. The abdominal wall and skin were closed in two layers using 1 vicryl interrupted sutures (polyglactin 910, Ethicon, Somerville, NJ, USA) in all, except the last protocol, or in three layers using EP6 Serafil (Serag-Wiessner, Naila, Germany) for the fascia, 2-0 vicryl for the subcutaneous tissue and 1 vicryl plus for the skin (skin protocol). Depomycin (20 mg/kg, subcutaneous, Intervet, Boxmeer, The Netherlands) was initiated preoperatively and maintained postoperatively for 3 or 5 days via intramuscular injections. In the last study the dosage was changed to 0.6 ml / 10kg and 1 ml / 10kg intramuscular, pre- and postoperative respectively.

Postoperative management.

To provide postoperative analgesia, buprenorphine (10 μg/kg, intravenous, Schering Plough, Segre, France) and flunixin (2 mg/kg, intramuscular) were given for three days in the last study on skin defects. In this study the ewes returned to the farm 1-2 hours after recovery accompanied by a 'buddy sheep' during their transport. In earlier studies, the animals stayed at the Central Animal Laboratory, RUNMC, Nijmegen, The Netherlands for 3-5 days. The surgical wound was sprayed every 2 days for 2 weeks with CTC spray (Eurovet Nederland, Bladel, The Netherlands) containing Chlortetracycline to prevent wound infection. Temperature, feeding behavior and wound control were monitored daily by the animal care takers from the Central Animal Laboratory and the farm. The animals stayed inside for several weeks (3-4 weeks depending on the season) before going outside to the meadow (Fig. 3).

Delivery and postnatal management.

In most studies cesarean delivery was performed under local anesthesia with lidocaïne (20-30 ml, 2% (v/v), subcutaneous and intramuscular, Fresenius Kabi, Bad Homburg, Germany). The ewes were euthanized as soon as the lambs were born.

In the latest study on bladder exstrophy, lambs were born vaginally. Parturition was induced at 140 days' gestation with dexadreson (12-15 ml, intramuscular, Intervet, Boxmeer, The Netherlands) and trilostane (Vetoryl, 60 mg, oral, Janssen-Cilag GmbH, Tilburg, The Netherlands). Labor started 36-48 hours later and was closely watched as the lambs were born a few days before term. The ewes were not sacrificed and gave breastfeeding during the first weeks.

For further evaluation, the newborn lambs were sacrificed immediately after birth or after one week depending on the protocol using medetomidine (0.5 mg, intramuscular, Orion pharma, Espoo, Finland) and pentobarbital (60 mg/kg, intracardial, AST Pharma, Oudewater, The Netherlands). In the studies on neural tube defects we performed also a functional surveillance of the lamb during one week besides a histological evaluation. In that period neurological tests were performed according to a standardized protocol. In the skin protocol, both ewes and fetal lambs were sacrificed at three time points (93, 107 and 140 days' gestation) using T61 (10 ml, intravenous; 5 ml, intracardial, Intervet, Boxmeer, The Netherlands).

Results

Outcomes for each study are summarized in table 1. In total, 172 surgical procedures were performed on fetal lambs divided over 12 different study protocols. Thirty seven sheep appeared to be not pregnant on ultrasonography prior to surgery and were excluded from the experiments. With our fertility protocol with intravaginal sponging we had a bearing incidence of 82% (163/200).

Miscarriage or stillbirth occurred in 44 cases. The overall fetal survival rate was 74% during the pregnancy period. Additional tests were performed concerning infections with Brucella melitensis, Toxoplasmosis or Chlamydia, however no infections with these specific micro-organisms could be found as a leading cause for miscarriage. In the study on amniotic fluid exchange, in most cases the microbiological cultures of the aborted lambs were positive for Streptococcus, Staphylococcus, Escherichia coli or Candida.

TABLE 1. OUTCOMES FOR EACH STUDY

Experiment	NTD covering		NTD AFE		Gastro schisis		Bladder exstrophy		Skin defect		All	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Total procedures	44		11		27		64		26		172	
Successful experiments	36	(82)	4	(36)	17	(63)	33	(52)	22	(85)	112	(65)
Fetal death	8	(18)	7	(64)	10	(37)	16	(25)	3	(12)	44	(26)
Maternal death	0		0		3	(11)	1	(2)	1	(6)	5	(3)
Complications	0		1	(9)	7	(26)	15	(23)	4	(15)	27	(16)

Numbers of operated *fetal lambs* are listed (n=172). Percentages are given in brackets and except for maternal death calculated as percentage of total procedures of operated lambs. Maternal death is calculated as percentage of total procedures of operated sheep (n=163) (NTD: neural tube defect, AFE: amniotic fluid exchange)

The experiment could be finished successfully in 65% of all experiments. Complications besides miscarriage or stillbirth are listed in table 2. In 22 experiments (13%) the complication led to death of the ewe and/or the lamb. In 4 cases, abdominal wound dehiscence in the ewe occurred after the surgical procedure necessitating euthanasia of the ewe. One ewe needed to be sacrificed because of severe diarrhea and at autopsy, herniation of a bowel loop between the fascial sutures was found. All complications in the ewe could be related to the laparotomy scar and did not occur after improvements of surgical technique. In the bladder exstrophy study with postnatal reconstruction, 4 experiments could not be finished because of a bowel herniation through the abdominal wall along the bladder exstrophy defect in the lamb. Three ewes delivered spontaneously before their planned cesarean section and the newborn lambs died immediately after birth. Four lambs died during or shortly after vaginal delivery. Postnatal cardiac failure, inflammation of the abdominal wall and a feeding problem of one lamb accounted for three postnatal deaths. One lamb died after the postnatal surgery of the bladder exstrophy. Inflammation, swelling of the wound and abdominal wound dehiscence with relaparotomy and survival of the ewes were seen as minor complications (n=5). One ewe delivered spontaneously which was unintended, but the lamb survived. One lamb developed a bowel inflammation

TABLE 2. SUMMARY OF COMPLICATIONS IN THE EWE OR IN THE LAMBS

	Description	n
Ewe	Euthanasia of the ewe subsequent to abdominal wound dehiscence	4
(n=9)	Death of the ewe due to herniation of a bowel loop between the fascial sutures	1
	Inflammation of the abdominal laparotomy wound and uneventful recovery	1
	Abdominal wound dehiscence with relaparotomy and uneventful recovery	2
	Swelling of the laparotomy wound with relaparotomy and uneventful recovery	1
Lamb	Death of the lamb due to bowel herniation along fetal bladder exstrophy defect	4
(n=18)	Postnatal death due to preterm delivery	3
	Death during birth	4
	Postnatal death due to cardiac failure	1
	Euthanasia of the lamb subsequent to postnatal inflammation of abdominal wall	1
	Postnatal death after neonatal bladder exstrophy surgery	1
	Postnatal death after 5 weeks due to a feeding problem	1
	Euthanasia of the lamb subsequent to severe urogenital problems	1
	Preterm labor and survival	1
	Bowel inflammation	1
	Total	27

without further consequence. In the skin defect protocol, a surgical procedure was performed on both lambs in case of twin pregnancy in 9 of 17 ewes which means a reduction in the use of laboratory animals of 35%. Keeping the ewe alive after vaginal delivery to re-use it in following experiments means an additional reduction of 100%.

Details on functional and histological outcome of the different studies were described previously [16-21].

Discussion

This paper describes our experience with different sheep models for fetal surgery over the past 10 years. Sheep were chosen in these experiments for different reasons. The anatomy is comparable to the human fetus and has a translational fetal development. The size of the fetal lamb at 79 days' gestation is comparable to the human fetus at 18-22 weeks' gestation and is sufficient to be suitable for complex fetal surgery [6]. Sheep are docile, tough and easy to handle [24]. During the experiment they can stay in their natural habitat. Because of the low rate of premature labor, no tocolytics are needed after intrauterine surgery. Myometrial contractions are present in pregnant sheep during fetal surgery but are suppressed by anesthesia. Inhalational agents provide uterine relaxation which allows handling of the uterus without contractions [25,26]. Anesthesia for the ewe is easy to induce and has a steady intra operative management without dangerously deep anesthetic levels and with a brief recovery period [24]. Complications due to anesthesia with this short duration of surgery did not occur within our studies. Additionally, there are a small number of fetuses present in each pregnancy and delivery induction is easy to control. Disadvantages of the use of sheep is the length of gestation (term, 140-147 days) which limits the number of experiments within a certain period of time and the experiments are expensive mainly due to the length of stay on the farm during the whole experiment and transport to the animal laboratory. Sheep mask pain, which makes assessment of postoperative pain difficult. Also, complete food deprivation prior to surgery is not possible. Several species (e.g. chicken, rat, rabbit, monkey, sheep) have been used to study the possible harmful effects of the intrauterine environment on congenital birth defects and the protective effect of fetal surgery [2,4,5,9,10,12,14,15,27-31]. Due to the many advantages, sheep are widely used as large animal model in experimental studies on fetal surgery. Of course, the surgically created defect is not completely comparable to the embryological defect in humans and the sheep model has its limitations, but it is reproducible and certainly valuable for testing treatment options such as new covering materials and techniques for fetal repair and fetal wound healing [3].

In total, 12 different studies with eight different surgical procedures have been performed, from which 172 fetal lambs and 163 ewes have been operated. Three studies on a neural tube defect, 2 of gastroschisis, 4 of bladder exstrophy and 3 on skin defects. After fetal surgery, 128 lambs survived pregnancy leading to an overall fetal survival of 74% which is consistent with the litera-

ture in which survival is documented ranging from 25 to 100% with a mean of 62% in all studies with fetal intervention [2,4,5,8-15].

The following improvements were made to the protocols.

Fertility protocol. To enhance pregnancy rate, crossbred between Swifter breed and Texel breed was used since the end of 2008 instead of Dutch Texel breed. The Swifter originally, is a crossbred between the Texel and the Flemish breeds. One of the characteristics and advantages of this breed is the high fertility and the better birth characteristics. In addition, by allowing the ram with the ewe on day thirteen instead of day fourteen, we try to improve pregnancy rate. In 2009, only one of the 28 sheep was not bearing (4% in comparison with 23% in 2008). In 2010, all 5 sheep were bearing.

Housing, handling and transport. In the latest studies, the ewes arrived at the Central Animal Laboratory a few hours prior to surgery and returned to the farm 1-2 hours after recovery, in contrary to previous studies, in which the animals stayed at the laboratory (at 21°C) for 3-5 days. For the welfare of the sheep, housing at the laboratory should be minimized and the animals should go back to their familiar environment as soon as possible (day case surgery). Because sheep are herd animals, the experiment animal was accompanied during their transport and/or stay by a 'buddy sheep' to enhance welfare and reduce stress for the animal. The operated sheep are housed separately for at least 3 to 4 weeks, but as such that the sheep still have nose to nose contact and can see and hear other sheep (Fig. 3A). The newborn lambs were housed with the mother for at least 1 or 2 months to reduce stress and to enable a proper follow-up of the operated sheep or lamb (Fig. 3B and C). The good condition of our bearing sheep, an uneventful postoperative course and the absence of feeding disorders are indicators of animal welfare in which the environment of the sheep plays an important role. Enhancement of welfare reduces stress and consequently keeps the animals in a better condition which is particularly important for an uneventful postoperative recovery. In addition, animals in stress eat less causing a digestional disorder which is a problem for ruminants.

Food deprivation. Sheep without a starvation period form gasses in their stomach and subsequent bowel dilatation which can cause problems during anesthesia and surgery. Because prolonged food deprivation of pregnant ewes will result in a negative energy balance which induces stress, the starvation period was reduced to a maximum of 16h. With the current protocols the sheep only eat dry grass hay 48h before surgery. In all cases the sheep had free access to water.

Anesthesia and analgesia. The methods of anesthesia and surgical procedures in the mother sheep had minor adaptations throughout the different experiments but did not change essentially. In the last study, propofol was given instead of pentobarbital for general anesthesia and flunixin and sufentanil to provide analgesia. Also buprenorphine and flunixin were given for three days to provide postoperative analgesia. Although probably the fetus perceives pain in a different way than in postnatal life, it is still necessary to provide analgesia and anesthesia to the fetus during fetal surgery [26,32]. Fetal analgesia for open fetal surgery is facilitated by maternal anesthesia. Lipophilic drugs, like isoflurane or propofol, can easily cross the placenta and in combination with opioids makes it suitable for combined mother/fetus anesthesia and are considered to provide adequate fetal anesthesia [33,34]. Studies in sheep suggests that the fetus requires a lower concentration of halothane to achieve the same level of anesthesia as the ewe does, so inhalational agents are considered to provide adequate fetal anesthesia [26,35]. After the neural tube defects studies, oxygen/nitrous oxide ventilation (O_2/N_2O) was replaced by oxygen in air ventilation (O_2/air) to prevent bloating of the stomach.

Surgery. Number of fetal deaths was highest in the neural tube defect exchange study, mainly caused by the high rate of infection by bacteria probably introduced by the intrauterine catheters. For this reason, a subcutaneous port-a-cath system (Deltec, Inc., St.Paul, USA) was used instead of catheters for sampling and exchange in two sheep in which no infection occurred. In the gastroschisis study, 3 maternal deaths occurred caused by an abdominal wound dehiscence of the ewe for which it had to be sacrificed accounting for 3 of the fetal deaths. Abdominal wound dehiscence was the major complication leading to maternal death because in all cases the ewe needed to be sacrificed as part of animal welfare. Therefore, the protocol considering closure of the abdominal wall and skin was improved. Instead of closing in two layers using 1 vicryl interrupted sutures, abdominal wall and skin were closed in three layers using EP6 Serafil for the fascia, 2-0 vicryl for the subcutaneous tissue and 1 vicryl plus for the skin. After this improvement, abdominal wound dehiscence with consequent euthanasia of the ewe was not seen since 2007. To prevent postoperative wound infection the surgical wound was sprayed every 2 days for 2 weeks with CTC spray (Eurovet Nederland, Bladel, The Netherlands) containing Chlortetracycline. With this preventive measure we haven't had any wound infection in the past two years.

In the bladder exstrophy protocol, we changed to a running suture technique to close the edges of the fetal bladder instead of interrupted sutures to prevent bowel herniation in the fetal lamb.

Delivery and postnatal care. In most studies, lambs were delivered by cesarean section under local anesthesia and the ewes were euthanized as soon as the lambs were born. In the skin protocol, ewes and fetal lambs were sacrificed together because a neonatal observational period of the lamb was not necessary and this method causes less inconvenience to the ewe. In the earlier studies our aim was to deliver all ewes by cesarean section but 4 animals had a preterm vaginal delivery. Three of these lambs died immediately after birth due to prematurity. Considering this small number, it is not our experience that ewes who have undergone fetal surgery labor a few days before expected term. Lambs were delivered by cesarean section because it is more controllable than vaginal delivery. In this way each lamb was born at exactly 140 days' gestation. In addition, we were concerned that a normal delivery could damage our surgical defects and thereby would influence the outcome. As an alternative, lambs were born vaginally after induction of parturition in the latest studies. In this way, the ewe doesn't have to be sacrificed and can be used in further studies. To prevent complications during delivery it is extremely important that delivery is closely observed by the animal care taker. After birth the mother sheep can give full attention and feed the newborn lambs, which is particularly important for the lambs with a defect. Newborn lambs receive mother's milk instead of bottle-feeding which also saves time for the animal care taker. In addition, we have positive experience with the re-use of earlier operated sheep for intrauterine surgery. This is also the case for the sibling, which has had no fetal surgery and can be used in further studies in later life. In this way, less laboratory animals are needed. Performing simple surgical procedures on both lambs in case of twin pregnancy (e.g. in the skin defect protocol) also contributed in reduction of the use of laboratory animals. At this moment we're developing an electronic registration for animal experiments similar as the digital patient file in hospitals. In this way all information about the animal, like surgical procedure, postoperative controls, food, medication and complications are kept in a central file which can be consulted by our researchers and animal care takers for information at any moment.

A summary of the most important improvements is listed in table 3.

TABLE 3. SUMMARY OF MOST IMPORTANT IMPROVEMENTS WITH THE RESULTS IN THE DIFFERENT PROTOCOLS

Protocol	Improvement	(expected) results
Fertility	Use of crossbred Swifter/Texel breed	Enhances fertility 96% pregnancy rate in 2009
Housing and transport	Minimize housing at the laboratory	Enhances welfare
	Buddy sheep during transport	Enhances welfare Reduces stress
	Housing of the newborns with the mother	Reduces stress Mother's milk instead of bottle feeding
	Electronic registration for animal experiments	Enhances quality of animal studies
Food	Reduction of the starvation period to 16h	Reduces stress
Anesthesia and analgesia	Postoperative analgesia	Enhances welfare Reduces stress
	O_2/air ventilation instead of O_2/N_2O	Prevents bloating of the stomach
Surgery	Closure of the abdominal wall in three instead of two layers	Reduces abdominal wound dehiscence with almost 100%
	Use of Chlortetracycline spray	Reduces postoperative wound infection with almost 100%
	Use of running suture to close the edges of the fetal bladder	Prevents bowel herniation
Delivery	Vaginal delivery after induction instead of SC	Reduces the use of animals with almost 100%
	Surgery on both lambs in case of twin pregnancy	Reduces the use of animals with 33%

Conclusions. The sheep models as described are well suited large animal models to study the reconstructive and regenerative properties of collagen scaffolds and tissue engineering techniques and the outcome of fetal and neonatal therapy for severe congenital birth defects. Besides use of laboratory animals, our fetal surgery group is also developing alternatives to *replace* animal models to study the properties of collagen scaffolds. With growing experience over the past 10 years and with *refinements* of the protocols the

number of animals can be *reduced* to gather the same results and the welfare of animals can be enhanced.

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CHAPTER 8

General discussion and future perspectives

General discussion

Spina bifida is a devastating condition with a substantial lifelong morbidity. Periconceptional folic acid supplementation reduces the risk as well as the recurrence risk of having a child with a neural tube defect and remains currently the only treatment to prevent neural tube defects. The introduction of routine ultrasonography screening programs allows the early, prenatal diagnosis of spina bifida. Early reliable prenatal detection of spina bifida offers opportunities for fetal repair which may be a viable alternative for prospective parents to the standard options of pregnancy termination or expective management. The MOMS trial, a large randomized trial of prenatal versus postnatal repair of spina bifida demonstrated that prenatal surgery for spina bifida reduced the need for a cerebrospinal fluid shunt and improved the degree of hindbrain herniation. Motor outcomes at 30 months were improved as compared with postnatal surgery. However prenatal therapy was associated with maternal and fetal risks. Premature rupture of membranes and preterm labor remains the 'Achilles heel' of fetal intervention and limits its effectiveness.

From the results of this thesis we can conclude that creation of an experimental neural tube defect in a chronic sheep model is feasible and long-term exposure of the unprotected spinal cord to the intrauterine environment can lead to damage of neural tissue and, consequently neurologic impairment. Coverage of the defect with a collagen scaffold can lead to a better neurologic outcome and can preserve the architecture of the spinal cord. Our findings provide further support for the practice of fetal surgery to prevent the secondary acquired damage to the spinal cord and strongly support the potential of tissue engineered collagen based constructs. The sheep model is a well suited large animal model to perform fetal interventions and to study the reconstructive and regenerative properties of collagen scaffolds and tissue engineering techniques for the intrauterine treatment of spina bifida. The collagen based scaffold used in this thesis offers good prospects for the use in fetal therapy.

Now the MOMS trial is finished and the first positive results are published, we should answer the question whether the potential benefits of fetal repair outweigh the fetal and maternal risks associated with in utero intervention. The introduction of fetal surgery for spina bifida in clinical practice raises important ethical questions which need to be addressed. In maternal-fetal surgery there are two patients and mother and fetus often have conflicting interests. Surgery for fetal therapy of spina bifida is also maternal surgery and the most important considerations in weighing the risks versus the benefits is the safety of the mother, her ability to have other children in the future and the risks in subsequent pregnancies. The potential benefits of fetal therapy must outweigh the maternal risks but a favorable risk-benefit ratio both for the woman and the fetus is difficult to determine. Originally, because of the maternal risks, fetal surgery was limited to lethal congenital anomalies. Although spina bifida can be devastating and severely disabling, in general it is not-life-threatening. The major potential benefit of fetal therapy is improvement in neurological outcome which may increase the probability of an independent life and this may justify fetal therapy. Especially when termination of pregnancy is no option for prospective parents, fetal surgery can be offered as a viable alternative instead of expective management until delivery.

Caution is necessary to start fetal therapy for spina bifida in many centers scattered around the world without making strict agreements. For years, fetal therapy for spina bifida in the US was only done in the three study centers. By diluting experience, fetal outcomes may not be as good as those in the MOMS trial and the maternal risks could be increased. The questions where these interventions should take place and by whom need to be addressed first. All involved maternal-fetal medicine specialists should have consensus about treatment and offering fetal therapy as an alternative to the patient before implementation. Fetal surgery should take place under strict conditions and the inclusion criteria should be specified and described in a protocol.

Although most of the complications like preterm premature rupture of membranes and preterm delivery are associated with open fetal surgery, also the endoscopic approach with the current techniques demonstrated an increased risk of preterm delivery, probably related to the membranous damage. In the MOMS trial, fetal repair of spina bifida was performed relatively late in gestation, after damage to the neural tissue probably has already

occurred. A less invasive and traumatic approach, applied earlier in gestation would theoretically increase the benefit and reduce the maternal and fetal risks. It should be emphasized that the positive outcomes of the MOMS trial cannot be simply translated to outcomes after endoscopic repair. Risks and benefits should also be evaluated in a randomized controlled trial comparing the current open approach to the endoscopic approach. Collagen based scaffolds offer good prospects for the use in endoscopic fetal therapy because they can be modulated and specially tailored to the needs of the endoscopic approach.

Future perspectives

Future studies should focus on early prenatal intervention with a minimally invasive approach and the use of tissue engineering techniques. Research should be done to develop smaller endoscopic instruments to reduce the risk of preterm delivery due to membranous damage. Smaller endoscopic instruments put high demands on the coverage materials. Future studies on collagen scaffolds should focus on those scaffolds which are eligible for application through an endoscope with a small diameter. Anticipating the endoscopic approach, the use of an easy applicable and biocompatible sealant to secure a scaffold to the defect should be taken into consideration to simplify and shorten the procedure.

Our study on skin defects demonstrated a beneficial effect of the use of scaffolds loaded with growth factors on fetal wound healing and incorporation of the scaffold in the surrounding tissue. The use of an advanced scaffold for fetal repair of spina bifida should also be evaluated in future animal studies. Postnatal follow up for 3 to 6 months is necessary to study the formation of adhesions and consequently tethered cord syndrome. Optimal concentration in which these growth factors need to be used still has to be established. Also the effects of growth factors other than VEGF and FGF2 can be evaluated.

Due to their reparative function and their role in the regenerative process, stem cells have emerged as a potential therapeutic modality for several diseases. Intrauterine coverage of spina bifida prevents further damage to the spinal cord but the spinal cord remains dysplastic. The hypothesis that the use of cell-seeded scaffolds or the application of stem cells to the site of the

defect as an adjuvant to fetal repair may promote regeneration of the dysplastic spinal cord and their role in fetal repair of spina bifida should be further evaluated.

Which collagen based scaffold has the best qualities to be used in fetal repair of spina bifida is still under evaluation in our studies. After the final conclusion, future studies should be directed to the use of a collagen based scaffold in a clinical trial and the application of tissue-engineered constructs in the human fetus.

Summary

Spina bifida results from failure of closure of the neural tube (neurulation) during early embryonic life and is one of the most common neural tube defects with a worldwide incidence of 1 in 2,000 live births. It is one of the most handicapping nonlethal congenital birth defects in humans, almost always leading to various problems with the lower extremities, bowel and bladder dysfunction, deformation of the lower extremities and back, sexual dysfunction, impaired mental development, hindbrain herniation and hydrocephalus. Children born with spina bifida suffer from lifelong disabilities and supportive care is often needed.

Improving folate status by folic acid supplementation reduces the risk as well as the recurrence risk of NTDs and periconceptional folic acid use should be encouraged. Advances in prenatal diagnosis and the use of routine ultrasonography screening programs now permit diagnosis of spina bifida as early as the first trimester. Early diagnosis makes spina bifida eligible for prenatal intervention and besides the choices of termination of pregnancy or expective management, fetal therapy can be seen as a third option for expectant parents. The rationale for in utero repair is the 'two-hit' hypothesis. Failure of primary neurulation in the embryonic period leads to the development of myelodysplasia (first-hit) and subsequently, the persistent exposure of the unprotected spinal cord to the intrauterine environment (e.g. amniotic fluid) can lead to secondary acquired destruction of neural tissue (second-hit) which may be prevented by in utero coverage of the defect.

From 2003 through December 2010, fetal surgery of spina bifida in humans was practiced in the USA in the three participating centers of the MOMS trial (Management of Myelomeningocele Study) in a randomized controlled trial comparing the two approaches of the treatment of children with spina bifida; fetal surgery and postnatal surgery. The long-expected results from this study were published in 2011. Prenatal surgery for spina bifida reduced

the need for shunting and improved motor outcomes at 30 months as compared with postnatal surgery.

Minimal access fetal surgery of spina bifida or coverage of larger defects requires the use of a degradable patch. Tissue engineering techniques have allowed the development of a scaffold, that can be used early in gestation in a minimally invasive approach. A biocompatible and biodegradable collagen scaffold offers good prospects for in utero coverage of a spina bifida and can protect the spinal cord against the intrauterine environment to prevent further neurological damage. The advantage of using such a tailor-made collagen scaffold is that with modulation of the matrix structure and the application of growth factors, cell attraction from the surrounding fetal tissue and wound healing can be enhanced.

Fetal therapy places the mother at risks for intraoperative complications and premature labor remains the 'Achilles heel' of open fetal surgery. Therefore, a less invasive approach is desirable. In addition, with an early endoscopic approach, damage to the spinal cord may be further prevented earlier in gestation to maximize preservation of neural tissue.

In this thesis, the development of an animal model is described, to study the use of tissue engineering techniques for the fetal treatment of spina bifida. The use of an advanced collagen matrix, loaded with growth factors, to improve fetal wound healing was evaluated. The feasibility of minimally invasive approaches, like endoscopy and amniotic fluid exchange for fetal therapy, was studied.

This thesis contains the following chapters:

In CHAPTER 1, the epidemiology, etiology, clinical outcome, prenatal diagnosis and current postnatal treatment of spina bifida is described. The rationale for in utero repair and the tissue engineering approach is discussed.

In CHAPTER 2 we evaluated whether acute intrauterine coverage of an experimental, surgically created, neural tube defect in fetal lambs could protect neural tissue from secondary injury during gestation and save neurologic functions after birth. We created a neural tube defect in fetal lambs at 79 days' gestation and covered it with either a molecular defined collagen-based biocompatible and biodegradable scaffold, a small intestinal submucosa (SIS)

biomatrix (Cook®) or by closing the skin over the defect. The outcome was compared to leaving the defect exposed to the intrauterine environment. All lambs with the defect covered were unimpaired while four of five neurologically examined lambs with the defect uncovered showed major neurologic impairment after birth. The results of this study demonstrated that creation of an experimental neural tube defect in a chronic sheep model is feasible and that long-term exposure of the open spinal cord to the intrauterine environment can lead to damage of neural tissue (second-hit) and, consequently, neurologic impairment. Acute coverage of the defect can lead to a better neurologic outcome. Both the collagen-based scaffold and the small intestinal submucosa biomatrix seem to be useful for in utero coverage of a surgically created neural tube defect in a sheep model.

In CHAPTER 3, the histological outcome of the study described in chapter 2 is assessed. The effect on the neural tissue of intrauterine coverage of an experimental neural tube defect in fetal lambs with two different biomatrices and the incorporation of the biomatrix in the surrounding tissues was determined. The outcome was compared to covering of the defect with skin or leaving the defect exposed to the intrauterine environment. All lambs with the defect uncovered showed histological abnormalities of the spinal cord without either signs of direct trauma attributable to the operation itself or recent trauma due to contact with the uterine wall nor signs of an inflammatory reaction. In lambs with the neural tube defect covered, one half showed a normal architecture of the spinal cord while minor histological damage was present in the other half. Between the three groups in which the defect was covered (collagen-based scaffold, a small intestinal submucosa (SIS) biomatrix (Cook®) or skin), the histological outcome was comparable. This study demonstrated that acute coverage of an experimental neural tube defect in fetal lambs prevents severe histological damage to the spinal cord and preserves the architecture of the spinal cord, independent of the two biomatrices used in this study. Long-term exposure of an unprotected spinal cord to the intrauterine environment can lead to severe damage of the spinal cord (second hit).

In CHAPTER 4 we evaluated whether the collagen scaffold, used in the previous studies, is useful for delayed in utero repair of a surgically created spina bifida in the fetal lamb and can protect the spinal cord from secondary injury. Instead of acute coverage, delayed repair was done for a better simulation of the natural course of spina bifida. Endoscopy in this study was performed

to achieve experience with the procedure in the sheep model and to study whether in utero endoscopic coverage is possible. In fetal lambs a spina bifida was created surgically at 72 or 79 days' gestation. The defect was covered with a collagen scaffold 2 weeks later or left uncovered. None of the surviving lambs with the defect covered showed loss of spinal function and the architecture of the spinal cord was preserved in 4 of the 5 lambs. Four of five lambs with the defect uncovered showed disturbance of the architecture. Due to technical difficulties with the endoscopic approach it was concluded that this model was less suitable to study the in utero endoscopic coverage of spina bifida. Collagen scaffolds can be used for delayed in utero covering of an experimental spina bifida and coverage preserves the architecture of the spinal cord. A major difference in neurological outcome between the 2 groups could not be demonstrated.

In CHAPTER 5 was investigated whether a collagen scaffold loaded with growth factors can be used to treat full-thickness fetal skin defects. Although most covered defects in our earlier studies were closed after birth, wound healing was not optimal. When collagen scaffolds are loaded with growth factors, angiogenesis and cellular infiltration are stimulated and consequently this may lead to an improved supply of oxygen and nutrients, which is particularly important when scaffolds of wider diameter are used. It was hypothesized that the pro-angiogenic growth factors VEGF and FGF2 might enhance vascularization and epithelialization, leading to improved wound healing. The study was performed to evaluate the biochemical properties of the collagen scaffold and the effects on fetal wound healing and for this reason only a fetal skin defect in a fetal sheep model was made. Three circular full-thickness skin defects were made on the back of fetal lambs at 79 days' gestation. Two lesions were covered with either a collagen scaffold or a collagen-heparin scaffold with VEGF and FGF2. One lesion was left uncovered. We evaluated fetal wound healing and closure of full-thickness wounds. All uncovered defects showed healing with scar formation and contraction. Compared to wounds treated with bare collagen scaffolds, wounds treated with growth factor-loaded scaffolds showed excessive formation of capillaries and less myofibroblasts, leading to less contraction. Interestingly, new appendages in the wounds treated with a collagen-heparin scaffold with VEGF and FGF2 were formed, 8 weeks after surgery. This study demonstrated that collagen scaffolds can be used to treat fetal skin defects and that the combination of collagen scaffolds with VEGF and FGF2 had a beneficial effect on wound healing.

In CHAPTER 6, the effect of amniotic fluid exchange on the concentrations of waste products in the amniotic fluid and the histological outcome in fetal sheep with a surgically created neural tube defect was evaluated. It is believed that both urinary waste products and gastrointestinal waste products are associated with neurotoxic effects on neural tissue. We hypothesized that amniotic fluid exchange is a minimally invasive approach that might prevent in utero neural tissue damage. The concentrations of urinary and gastrointestinal waste products in the amniotic fluid before and after exchange were measured. An experimental neural tube defect was created in fetal lambs and left uncovered. In one group only samples of amniotic fluid were collected at a regular interval and analyzed for assay of waste products. In the other group also amniotic fluid exchange was performed. Urinary and gastrointestinal waste product concentrations in the amniotic fluid return to normal within one week after amniotic fluid exchange. From these results we concluded that a frequency of amniotic fluid exchange of one week is too low to prevent neural tissue damage. In both groups, all lambs showed major histological abnormalities of the spinal cord and a reduction of neural tissue damage could not be demonstrated. Since the study groups were very small, no definite conclusions can be drawn from the histological evaluations. However, this study indicates that in utero coverage is a more realistic approach of fetal therapy of spina bifida than amniotic fluid exchange.

In CHAPTER 7, an overview is provided of the animal models (pregnant sheep and fetal lamb) which we developed in the past 10 years to study fetal surgery and the use of tissue engineering techniques for the treatment of congenital birth defects (spina bifida, gastroschisis, bladder exstrophy) and an experimental skin defect, in the pre- and postnatal period. Since application of a collagen scaffold in humans is not technically or ethically possible at this moment, animal models are indispensable. According to the 3 R's (replacement, refinement and reduction), adaptations to the protocols were made for re-use (reduction) and refinement to improve animal welfare. One hundred and seventy two surgical procedures were performed in fetal lambs divided over 12 different study protocols. The overall fetal survival rate was 74% during the pregnancy period and the experiment could be finished successfully in 65% of all the cases. From our experience we concluded that the sheep model is a well suited large animal model to perform fetal interventions and to study the reconstructive and regenerative properties of collagen scaffolds and tissue engineering techniques for the treatment of severe congenital

birth defects. With the achieved experience over the past 10 years and with refinements of the protocols, the number of animals can be reduced and the welfare of the laboratory animal be enhanced.

In CHAPTER 8, a general discussion and future perspectives are given. With this thesis we provide further evidence for the 'two-hit' hypothesis of spina bifida. Intrauterine coverage of the defect with a collagen scaffold preserves the architecture of the spinal cord and the incorporation of growth factors has a beneficial effect on fetal wound healing.

Future studies on fetal repair of spina bifida should focus on minimally invasive techniques applied early in pregnancy, with the use of tissue engineering techniques. Ethical issues concerning fetal therapy for spina bifida and the use of tissue engineering techniques in the human fetus are discussed.

Samenvatting

Een spina bifida is een sluitingsdefect van de neuraalbuis en is een van de meest voorkomende aangeboren afwijkingen van het centrale zenuwstelsel. De afwijking ontstaat al vroeg in de zwangerschap tijdens de embryonale periode. De wereldwijde incidentie bedraagt 1 op de 2000 levendgeborenen. Spina bifida is een van de meest invaliderende, niet-letale aangeboren afwijkingen en kan aanleiding geven tot verlammingsverschijnselen en sensibiliteitsstoornissen van de onderste extremiteiten, disfunctioneren van darm en blaas, vergroeiingen van de benen en de rug, sexuele functiestoornissen, verstandelijke handicaps, hydrocephalus en een inklemming van de kleine hersenen en hersenstam (Chiari II malformatie). Door de handicaps zijn patiënten vaak levenslang afhankelijk van medische en sociale zorg en ondersteuning.

Periconceptioneel gebruik van foliumzuur verlaagt zowel het risico als het herhalingsrisico op neuraalbuisdefecten en het gebruik in de periconceptionele fase moet worden aangemoedigd. Verbeteringen in de prenatale screening en diagnostiek en de invoering van het structureel echoscopisch onderzoek bij iedere zwangere vrouw, maken een vroege diagnose mogelijk, soms zelfs al in het eerste trimester. Een vroegtijdige diagnose van spina bifida biedt naast de keuzes van afbreking van de zwangerschap of een expectatief beleid, de mogelijkheid van foetale interventie om de uitkomst van kinderen, geboren met een spina bifida, te verbeteren. De ratio achter de toepassing van intra-uteriene behandeling van spina bifida is de zogenaamde '*two-hit*' hypothese. Door een sluitingsdefect in de neuraalbuis is het ruggenmerg niet goed aangelegd en wordt niet beschermd door bot en omliggende weefsels. Het zenuwweefsel staat ten gevolge hiervan blootgesteld aan de toxische en mechanische schadelijke invloeden van het intra-uteriene milieu zoals het vruchtwater. Deze schadelijke invloeden kunnen leiden tot verdere beschadiging van een, in aanleg al, afwijkend ruggenmerg. De '*first-hit*' is de embryonale stoornis waarbij er een dysplastisch ruggenmerg gevormd wordt. De intra-uterien verworden, secundaire schade wordt de '*second-hit*' genoemd.

Van 2003 tot december 2010 werd foetale behandeling van spina bifida in de Verenigde Staten verricht in de drie deelnemende centra aan de "MOMS trial" (*Management Of Myelomeningocele Study*), een gerandomiseerde multicenter studie waarbij de uitkomsten na foetale behandeling vergeleken werden met de uitkomsten na standaard postnatale behandeling. De langverwachte uitkomsten van deze studie werden gepubliceerd in 2011. De resultaten laten een daling zien van het aantal plaatsingen van een shunt voor de behandeling van hydrocephalus na de geboorte na foetale behandeling en een verbetering van de motoriek op de leeftijd van 30 maanden in vergelijking met postnatale behandeling.

Foetale behandeling van grote defecten of een endoscopische benadering maakt het gebruik van een biologisch afbreekbare patch als afdekmateriaal wenselijk. Door gebruik te maken van weefseltechnologie wordt het mogelijk om een matrix te ontwikkelen die vroeg in de zwangerschap op een minimaal invasieve manier aangebracht kan worden. Een biocompatibele en biologisch afbreekbare collageenmatrix is goed toepasbaar bij het prenataal bedekken van een spina bifida en beschermt het zenuwweefsel tegen de intra-uteriene omgeving om verdere neurologische schade te voorkomen. Een van de voordelen van de toepassing van een collageenmatrix is, dat door aanpassing van de driedimensionale structuur en het aanbrengen van groeifactoren, het aantrekken van cellen uit het omliggende weefsel en wondgenezing kan worden bevorderd.

Foetale behandeling stelt de zwangere vrouw bloot aan intra-operatieve complicaties en het risico op vroeggeboorte blijft het grootste probleem van open foetale chirurgie waarbij een hysterotomie wordt verricht. Een minimaal invasieve ingreep, zoals een endoscopische benadering zou daarom de voorkeur hebben. Een endoscopische behandeling zou verder als voordeel hebben dat deze in een vroeger stadium in de zwangerschap verricht kan worden waardoor secundaire schade aan het ruggenmerg door de intra-uteriene omgeving eerder voorkomen kan worden.

In dit proefschrift wordt de ontwikkeling van een diermodel beschreven om de toepassing van weefseltechnologie bij de foetale behandeling van spina bifida te kunnen onderzoeken. Door gebruik te maken van een geavanceerde collageenmatrix met groeifactoren werd onderzocht of de foetale wondgenezing kan worden verbeterd. Minimaal invasieve benaderingen zoals, endoscopie en vruchtwaterwisselingen, werden geëvalueerd op hun toepasbaarheid in de foetale therapie.

Het proefschrift is onderverdeeld in de volgende hoofdstukken:

In HOOFDSTUK 1 wordt een overzicht gegeven van de epidemiologie, etiologie, symptomatologie, prenatale diagnostiek en huidige postnatale behandeling van spina bifida. De foetale behandeling van spina bifida en de toepassing van weefseltechnologie wordt besproken.

In HOOFDSTUK 2 worden de resultaten beschreven van een studie waarin werd onderzocht of directe intra-uteriene bedekking van een experimenteel, chirurgisch aangelegde spina bifida in het foetale lam het neurale weefsel kan beschermen tegen de secundaire schade van de intra-uteriene omgeving en normale neurologische functies na de geboorte behouden kunnen worden. In het foetale lam werd bij 79 dagen zwangerschapsduur een neuraalbuis defect gemaakt en gesloten met, een moleculair gedefinieerde, biocompatibele en biologisch afbreekbare collageenmatrix, een SIS matrix (vervaardigd uit dunne darm submucosa, Cook®) of met het sluiten van de huid over het defect. Alle lammeren waarbij het defect tijdens de zwangerschap werd gesloten lieten geen neurologische uitval zien terwijl vier van de vijf neurologisch onderzochte lammeren met een niet afgedekt defect ernstige neurologische afwijkingen hadden na de geboorte. De resultaten van deze studie laten zien dat het aanleggen van een experimenteel neuraalbuisdefect in een chronisch schapenmodel mogelijk is en dat langdurige blootstelling van het niet afgedekte ruggenmerg aan de intra-uteriene omgeving kan leiden tot schade aan het zenuwweefsel (de zogenaamde '*second-hit*') wat neurologische uitval tot gevolg heeft. Directe sluiting van het defect geeft een betere neurologische uitkomst. Zowel de collageenmatrix als de SIS matrix zijn beiden bruikbaar voor het intra-uterien sluiten van een chirurgisch aangelegd neuraalbuis defect in een schapenmodel.

In HOOFDSTUK 3 worden de histologische uitkomsten beschreven van de studie genoemd in hoofdstuk 2. De effecten op het zenuwweefsel na het intra-uterien bedekken van een experimenteel neuraalbuis defect met twee verschillende matrices en de incorporatie in het omliggende weefsel werden onderzocht in het foetale lam. De uitkomsten werden vergeleken met lammeren waarbij primair de huid over het defect werd gesloten en lammeren waarbij het defect onbedekt werd gelaten. Alle lammeren waarbij het defect niet werd afgedekt laten histologische afwijkingen zien van het ruggenmerg. Er zijn geen aanwijzingen van direct trauma veroorzaakt door de operatie zelf of door contact met de wand van de uterus, of tekenen van een ontste-

kingsreactie. In lammeren waarbij het defect werd afgedekt liet de helft een normale architectuur van het ruggenmerg zien. In de andere helft werden geringe histologische veranderingen gevonden. Tussen de drie verschillende groepen onderling (collageenmatrix, SIS matrix en huid), was de histologische uitkomst vergelijkbaar. Deze uitkomsten laten zien dat het direct bedekken van een experimenteel neuraalbuis defect in het foetale lam, ernstige histologische schade aan het ruggenmerg kan voorkomen en de architectuur van het ruggenmerg kan behouden. Langdurige blootstelling van het niet afgedekte zenuwweefsel aan de intra-uteriene omgeving kan leiden tot ernstige schade van het ruggenmerg. De uitkomsten zijn onafhankelijk van de gebruikte matrix.

In HOOFDSTUK 4 wordt een studie beschreven waarin werd onderzocht of de eerder gebruikte collageenmatrix ook bruikbaar is voor het afdekken van een chirurgisch gecreëerde spina bifida in het foetale lam, twee weken nadat het is aangelegd, en het ruggenmerg kan beschermen tegen secundaire schade. In tegenstelling tot de eerdere studies waarbij het defect direct tijdens dezelfde ingreep werd aangelegd, werd hier gekozen voor het twee weken later afdekken van de laesie om het natuurlijke beloop van een spina bifida beter te simuleren. In deze studie werd tevens gebruik gemaakt van een endoscopische benadering om ervaring op te doen met de procedure en te onderzoeken, of het aanbrengen van een matrix via de endoscopische weg mogelijk is. In het foetale lam werd chirurgisch een spina bifida aangelegd bij 72 of 79 dagen zwangerschapsduur. Het defect werd twee weken later gesloten met een collageenmatrix of onbedekt gelaten. Geen van de overlevende lammeren met een gesloten defect liet verlies van neurologische functies zien en de architectuur van het ruggenmerg bleef behouden in 4 van de 5 lammeren. Vier van de vijf lammeren met het niet afgedekte defect, lieten veranderingen zien van de architectuur van het ruggenmerg. Hieruit kon geconcludeerd worden dat een collageenmatrix ook bruikbaar is voor het afdekken van een chirurgisch gecreëerde spina bifida twee weken na het aanleggen van het defect. Na afdekken blijft de architectuur van het ruggenmerg behouden. Een groot verschil in neurologische uitkomst tussen de twee groepen kon niet worden aangetoond. Door de moeilijkheden met de endoscopische benadering in deze studie, kon geconcludeerd worden dat dit model minder geschikt is om onderzoek te doen naar het endoscopisch bedekken van het defect.

In HOOFDSTUK 5 wordt een studie beschreven waarin de toepasbaarheid werd onderzocht van het intra-uterien behandelen van een huiddefect met

een collageenmatrix beladen met groeifactoren. Bij de eerder beschreven studies waarin defecten intra-uterien werden bedekt met een collageenmatrix zonder groeifactoren, bleek de huid gesloten te zijn bij de geboorte maar het wondgenezingsproces was niet optimaal. Wanneer een collageenmatrix beladen wordt met groeifactoren, wordt de angiogenese en cellulaire infiltratie bevorderd. Dit heeft een positief effect of de aanvoer van zuurstof en voedingsstoffen wat vooral van belang is wanneer een matrix met een grotere diameter wordt gebruikt. De hypothese dat de pro-angiogene groeifactoren VEGF en FGF2, wondgenezing zouden kunnen verbeteren door hun stimulerende effect op vascularisatie en epithelisatie werd in deze studie onderzocht. In deze studie werd alleen gekeken naar de biochemische eigenschappen van de collageenmatrix en de effecten op de foetale wondgenezing en om die reden werd het onderzoek beperkt tot het aanleggen van alleen een huiddefect. Bij een zwangerschapsduur van 79 dagen werden drie circulaire defecten aangelegd in de volledige huidlaag op de rug bij het foetale lam. Twee van de drie defecten werden gesloten. Eén met een collageenmatrix zonder en één met een collageen-heparine matrix met de groeifactoren VEGF en FGF2. Het derde defect werd niet gesloten. Wondgenezing en sluiting van de wond werden geëvalueerd. Alle defecten die niet werden gesloten lieten wondgenezing met littekenformatie en contractie zien. In vergelijking met de defecten met een collageenmatrix, werd in de defecten met een collageen-heparine matrix met groeifactoren, uitgebreide nieuwvorming van capillairen gevonden en waren er minder myofibroblasten aanwezig en dientengevolge minder contractie. In de defecten die werden gesloten met een collageen-heparine matrix met groeifactoren werden, 8 weken na chirurgie, zelfs nieuwe huidappendices gevonden. De resultaten laten zien dat een collageenmatrix toepasbaar is bij het sluiten van een foetaal huiddefect en dat een collageenmatrix beladen met de groeifactoren VEGF en FGF2 een positief effect op de wondgenezing laat zien.

In HOOFDSTUK 6 wordt een studie beschreven waarin het effect van vruchtwaterwisselingen op de concentratie van uitscheidingsproducten in het vruchtwater en op de histologische uitkomst van een chirurgisch aangelegde spina bifida in het foetale lam werd onderzocht. De in het vruchtwater aanwezige, urinaire en gastro-intestinale uitscheidingsproducten hebben mogelijk een neurotoxisch effect op het zenuwweefsel bij spina bifida. Onze hypothese is dat vruchtwaterwisseling als minimaal invasieve benadering, intra-uteriene schade aan het ruggenmerg bij spina bifida kan voorkomen. Hiervoor werd chirurgisch een neuraalbuis defect aangelegd in foetale lam-

meren wat niet werd afgedekt. De concentraties van de urinaire en gastro-intestinale uitscheidingsproducten in het vruchtwater werden gemeten voor en na wisseling. In één groep werden op regelmatige tijdstippen alleen monsters genomen van het vruchtwater en geanalyseerd op de aanwezigheid van uitscheidingsproducten. In de andere groep vond ook wisseling van het vruchtwater plaats. Concentraties van urinaire en gastro-intestinale uitscheidingsproducten in het vruchtwater keerden binnen een week terug naar hun normaalwaarden, waaruit geconcludeerd kon worden dat een frequentie van vruchtwaterwisselingen van één week te laag is om schade aan het zenuwweefsel te voorkomen. In beide groepen werden bij alle lammeren ernstige histologische afwijkingen aan het ruggenmerg gevonden en kon schade aan het ruggenmerg niet worden voorkomen door het wisselen van vruchtwater. Beide studiegroepen zijn echter te klein in aantal om definitieve conclusies te trekken uit de histologische uitkomsten. Deze studie laat echter wel zien dat het intra-uterien bedekken een realistischer vorm van foetale behandeling van spina bifida is dan vruchtwaterwisseling.

In HOOFDSTUK 7 wordt een overzicht gegeven van alle diermodellen (drachtig schaap en foetale lam) die wij in de afgelopen 10 jaar hebben ontwikkeld om foetale chirurgie en de toepassing van weefseltechnologie bij de behandeling van aangeboren afwijkingen (spina bifida, gastroschisis, blaasexstrofie) en een experimenteel huiddefect in de pre- en postnatale periode te onderzoeken. Onderzoek van deze technologie en de toepassing van een collageen matrix blijft vooralsnog noodzakelijk in diermodellen omdat toepassing in mensen technisch en ethisch gezien nog niet mogelijk is. Met als uitgangspunt de 3V's voor dierproeven (vervanging, vermindering en verfijning) zijn de protocollen de afgelopen jaren aangepast, in het bijzonder op het gebied van hergebruik van proefdieren en bevordering van het welzijn.
In totaal zijn er 172 chirurgische procedures verricht in foetale lammeren verdeeld over 12 verschillende onderzoeksprotocollen. De foetale overleving tot aan de geboorte bedroeg 74%. Vijfenzestig procent van alle experimenten kon succesvol worden afgerond. Vanuit deze ervaringen kunnen wij concluderen dat het schapenmodel een zeer geschikt, groot diermodel is om foetale interventies op uit te voeren en de reconstructieve en regeneratieve mogelijkheden van een collageenmatrix voor de behandeling van aangeboren afwijkingen te onderzoeken. Met onze ervaring die we hebben opgedaan in de afgelopen 10 jaar en met verfijning van de onderzoeksprotocollen hebben wij het gebruik van proefdieren in onze studies kunnen verminderen en het welzijn voor de proefdieren kunnen verhogen.

In HOOFDSTUK 8 worden een algemene discussie beschreven en perspectieven voor de toekomst genoemd. De uitkomsten van dit proefschrift geven een verder bewijs voor het bestaan van de '*two-hit*' hypothese bij spina bifida. Intra-uterien afdekken van het defect met een collageenmatrix geeft behoud van de architectuur van het ruggenmerg en de toevoeging van groeifactoren aan de collageenmatrix kan de wondgenezing verbeteren. Toekomstig onderzoek naar de foetale behandeling van spina bifida dient zich te richten op een minimaal invasieve benadering, vroeg in de zwangerschap, daarbij gebruik makend van weefseltechnologie. De ethische vraagstukken met betrekking tot foetale therapie bij spina bifida en de toepassing van weefseltechnologie bij de humane foetus worden besproken.

Abbreviations

ABC	Avidine-biotine complex
AEC	3-amino-5-ethylcarbazole
AF	Amniotic fluid
AFE	Amniotic fluid exchange
CM	Chiari II malformation
COL	Collagen scaffold
COL-HEP/VF	Collagen scaffold loaded with VEGF and FGF2
CSF	Cerebrospinal fluid
DAB	Diaminobenzidine
ECM	Extracellular matrix
EDC	1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide
EuroSTEC	Integrated project on 'Soft tissue engineering for congenital birth defects in children'
EVG	Elastin Van Gieson
FBR	Foreign body reaction
FGF2	Basic fibroblast growth factor
GGT	Gamma-glutamyltransferase
HE	Hematoxylin/eosin
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
MES	2-morpholinoethane sulfonic acid
MMC	Myelomeningocele
MOMS	Management Of Myelomeningocele Study
NHS	N-hydroxysuccinimide
NTD	Neural tube defect
NTDs	Neural tube defects
PBS	Phosphate buffered saline
PMSG	Pregnant mare serum gonadotropin
RR	Relative risk
RU-DEC	Local Ethics Committee on Animal Research of the Radboud University Nijmegen

RUNMC	Radboud University Nijmegen Medical Centre
SB	Spina bifida
SBA	Spina bifida aperta
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SIS	Small intestinal submucosa
TE	Tissue engineering
UMC	University Medical Centre Nijmegen
VEGF	Vascular endothelial growth factor
α-SMA	α-smooth muscle actin

Authors

From the Department of Obstetrics and Gynecology, University Medical Centre Nijmegen, The Netherlands: *F.K. Lotgering and A.J. Crevels.*

From the Department of Obstetrics and Gynecology, University Medical Centre Groningen, The Netherlands: *P.P. van den Berg.*

From the Department of Urology, University Medical Centre Nijmegen, The Netherlands: *W.F.J. Feitz, L.A.J. Roelofs, P.J. Geutjes, E. Oosterwijk and D.M. Tiemessen.*

From the Departments of Pediatric Surgery, University Medical Centre Nijmegen, The Netherlands and Erasmus MC Sophia Children's Hospital Rotterdam, The Netherlands: *R.M.H. Wijnen.*

From the Department of Biochemistry, University Medical Centre Nijmegen, The Netherlands: *T.H. van Kuppevelt, W.F. Daamen and H.T.B. van Moerkerk.*

From the Department of Pathology, University Medical Centre Nijmegen, The Netherlands: *M.M.Y. Lammens, W.A.M. Blokx and K. Verrijp.*

From the Department of Pathology and Medical Biology, University Medical Centre Groningen, The Netherlands: *N.A. Hosper, M.J.A. van Luyn, R.A. Bank and M.C. Harmsen.*

From the Department of Pediatric Neurology, University Medical Centre Nijmegen, The Netherlands: *R.A. Mullaart.*

From the Department of Neurosurgery, University Medical Centre Nijmegen, The Netherlands: *J.A. Grotenhuis.*

From the Central Animal Laboratory, University Medical Centre Nijmegen, The Netherlands: *A.E.J. Hanssen and C. van den Broek.*

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Curriculum vitae

Alex Eggink werd geboren op 8 september 1968 in Rotterdam. Na het behalen van zijn VWO diploma in 1986 aan de Scholengemeenschap Johannes Calvijn te Rotterdam, studeerde hij geneeskunde aan de Erasmus Universiteit Rotterdam.
In 1993 haalde hij zijn artsexamen en vervulde hij daarna de militaire dienstplicht als eerste luitenant-arts bij het legerkorps op de militaire basis in Ede.
Aansluitend in 1994 werkte hij 1 jaar als ANIOS in het IJsselland Ziekenhuis in Capelle aan den IJssel (hoofd J.Kal). In 1995 verhuisde hij naar Nijmegen en werkte als ANIOS tot en met 1997 in het Canisius Wilhelmina Ziekenhuis in Nijmegen (opleider dr. W.B.K.M.V. de Goeij).
In 1998 begon hij met de opleiding tot gynaecoloog in het cluster Nijmegen. Naast het Universitair Medisch Centrum St Radboud in Nijmegen (opleiders prof. dr. J.M.W.M. Merkus en prof. dr. H. Boonstra†) volgde hij het perifere deel van zijn opleiding in het Catharina Ziekenhuis in Eindhoven (opleider dr. P.A. van Dop). Na afronding van zijn opleiding begon hij in juli 2003 met zijn fellowship Perinatologie op de afdeling Verloskunde en Gynaecologie van het Universitair Medisch Centrum St Radboud in Nijmegen en startte hij gelijktijdig met het promotieonderzoek bij prof. dr. W.F.J. Feitz, prof. dr. P.P. van den Berg en prof. dr. F.K. Lotgering. In deze periode sloot hij zich aan bij de projectgroep "Foetale Chirurgie", een multidisciplinair samenwerkingsverband tussen de afdelingen Verloskunde en Gynaecologie, Kinderurologie, Kinderchirurgie en Biochemie van het Universitair Medisch Centrum St Radboud in Nijmegen wat de mogelijkheden onderzoekt van de toepassing van tissue engineering bij de behandeling van aangeboren afwijkingen in het diermodel.
Na zijn fellowship perinatologie was hij vanaf 1 juli 2005 tot 1 april 2011 werkzaam als staflid op de afdeling Verloskunde en Gynaecologie van het Universitair Medisch Centrum St Radboud in Nijmegen met bijzondere interesse in de prenatale diagnostiek en therapie. Naast de echoscopische diagnostiek van aangeboren afwijkingen had hij een actieve rol in de implementatie van nieuwe cytogenetische technieken in de prenatale diagnostiek. Het laatste

jaar was hij tevens werkzaam als deskundige prenatale screening bij de Stichting Prenatale screening regio Nijmegen (SPN).
Op 1 april 2011 keerde hij terug naar zijn geboortestad en zette hij zijn carrière als gynaecoloog voort op de afdeling Verloskunde en Gynaecologie van het Erasmus Medisch Centrum in Rotterdam.
Alex Eggink is getrouwd met Eva Maria Roes en samen hebben zij een dochter Carolien.

PubMed links

In utero repair of an experimental neural tube defect in a chronic sheep model using biomatrices

Histological evaluation of acute covering of an experimental neural tube defect with biomatrices in fetal sheep

Delayed intrauterine repair of an experimental spina bifida with a collagen biomatrix

Intrauterine tissue engineering of full-thickness skin defects in a fetal sheep model

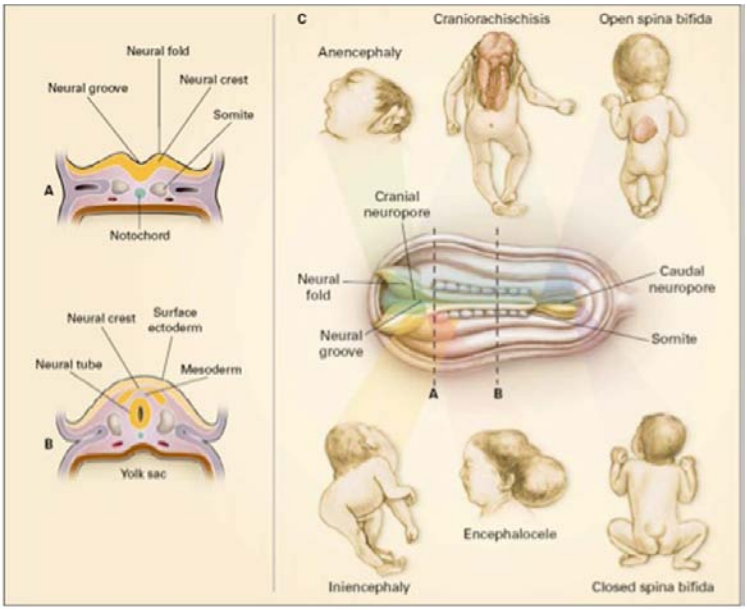

FIG. 1. Features of neural tube development and neural tube defects.
Panel A shows a cross section of the rostral end of the embryo at approximately three weeks after conception, showing the neural groove in the process of closing, overlying the notochord. The neural folds are the rising margins of the neural tube, topped by the neural crest, and demarcate the neural groove centrally.
Panel B shows a cross section of the middle portion of the embryo after the neural tube has closed. The neural tube, which will ultimately develop into the spinal cord, is now covered by surface ectoderm (later, the skin). The intervening mesoderm will form the bony spine. The notochord is regressing.
Panel C shows the developmental and clinical features of the main types of neural-tube defects. The diagram in the center is a dorsal view of a developing embryo, showing a neural tube that is closed in the center but still open at the cranial and caudal ends. The dotted lines marked A and B refer to the cross sections shown in Panels A and B. Shaded bars point to the region of the neural tube relevant to each defect. In anencephaly, the absence of the brain and calvaria can be total or partial. Craniorachischisis is characterized by anencephaly accompanied by a contiguous bony defect of the spine and exposure of neural tissue. In open spina bifida, a bony defect of the posterior vertebral arches (in this case, the lower thoracic vertebrae) is accompanied by herniation of neural tissue and meninges and is not covered by skin. In iniencephaly, dysraphia in the occipital region is accompanied by severe retroflexion of the neck and trunk. In encephalocele, the brain and meninges herniate through a defect in the calvaria. In closed spina bifida, unlike open spina bifida, the bony defect of the posterior vertebral arches (in this case, the lumbar vertebrae), the herniated meninges, and neural tissue are covered by skin. Figure reproduced with permission from The New England Journal of Medicine [7].

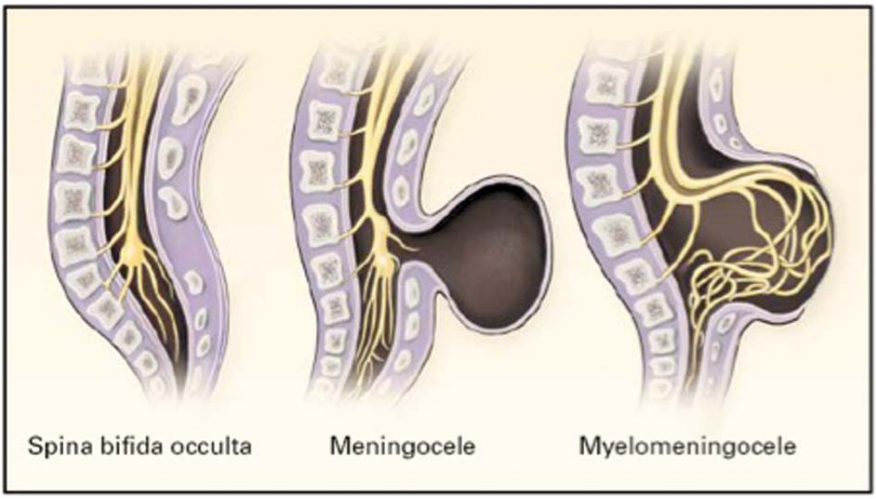

FIG. 2. Lateral view of the spinal cord in three types of spina bifida. Spina bifida occulta occurs most often at S1, S2, or both and is a bony defect of the spine, usually covered by normal skin. A meningocele is a saccular herniation of meninges and cerebrospinal fluid through a bony defect of the spine. Meningoceles are usually covered by normal skin. A myelomeningocele is the most common type of spina bifida and is characterized by herniation of the spinal cord, nerves, or both through a bony defect of the spine. Myelomeningoceles are usually open defects in which either meninges or neural tissue is exposed to the environment. Of these three types, only meningocele and myelomeningocele are typically included in studies of spina bifida and are often jointly referred to as spina bifida cystica. The spinal cord and nerves are depicted in yellow and the cerebrospinal fluid is in black. Figure reproduced with permission from The New England Journal of Medicine [7].

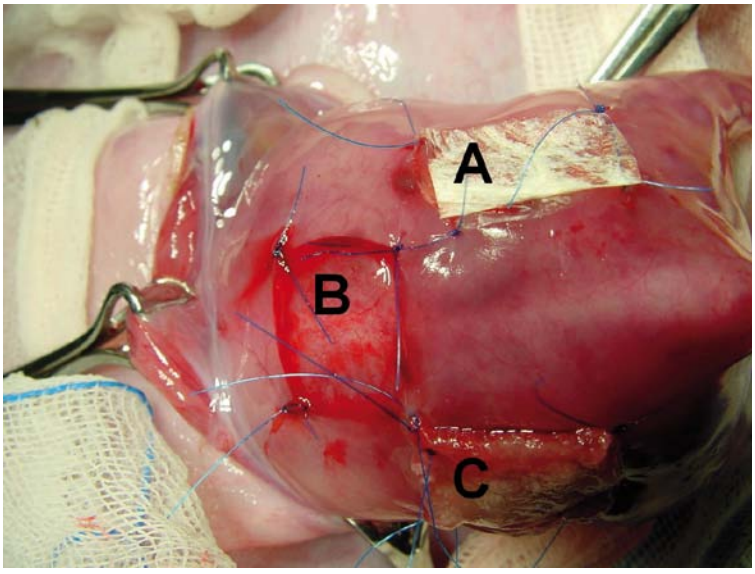

FIG. 1. Sham control with skin lesions. The lesion on the right side is covered with the SIS biomatrix (A), the left side is covered with the UMC biomatrix (C) and the lesion in the middle remains uncovered (B).

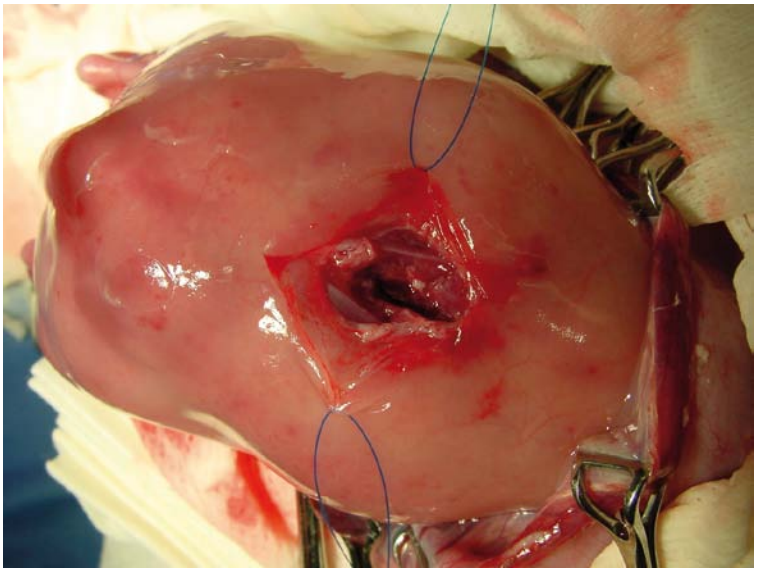

FIG. 2. Surgically created neural tube defect.

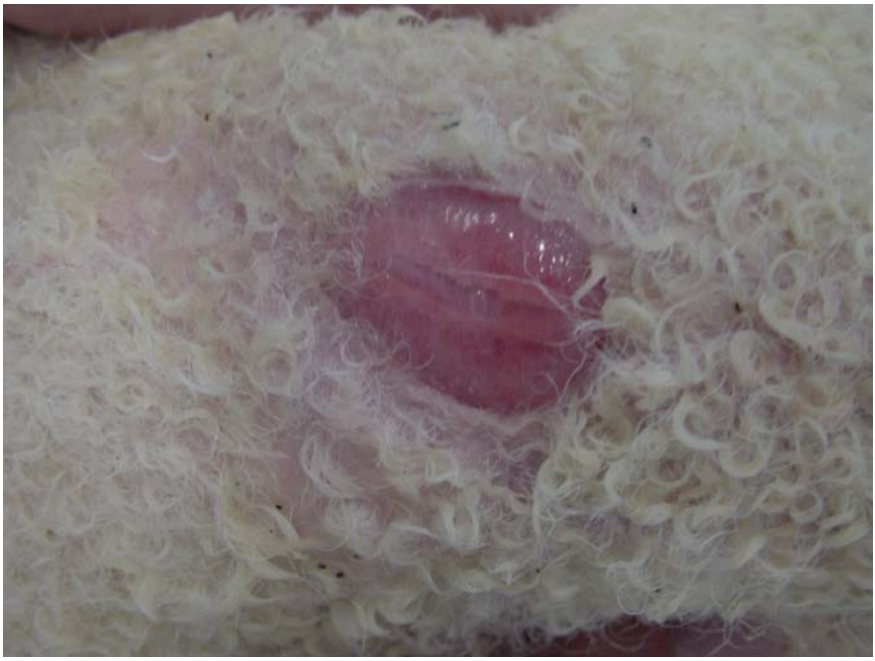

FIG. 3. Neonatal lamb with surgically created neural tube defect.

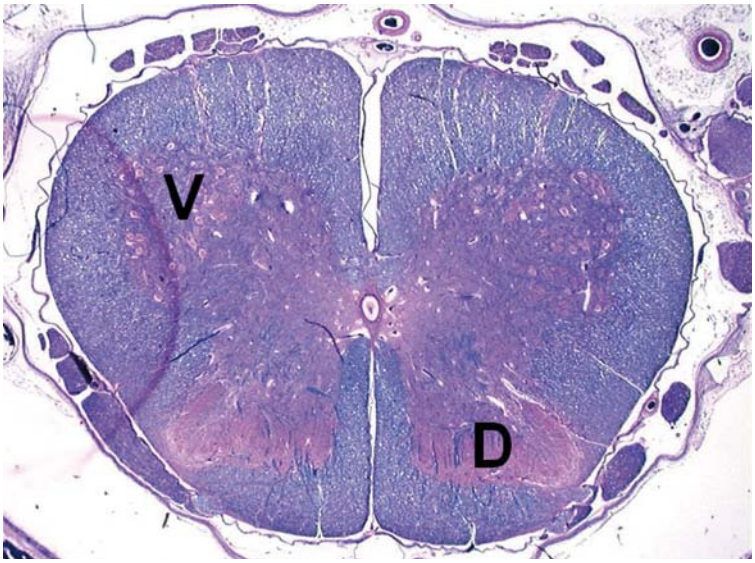

FIG. 2. Cross-section (HE staining) of the normal spinal cord of a sham-operated control. D = Dorsal horn; V = ventral horn.

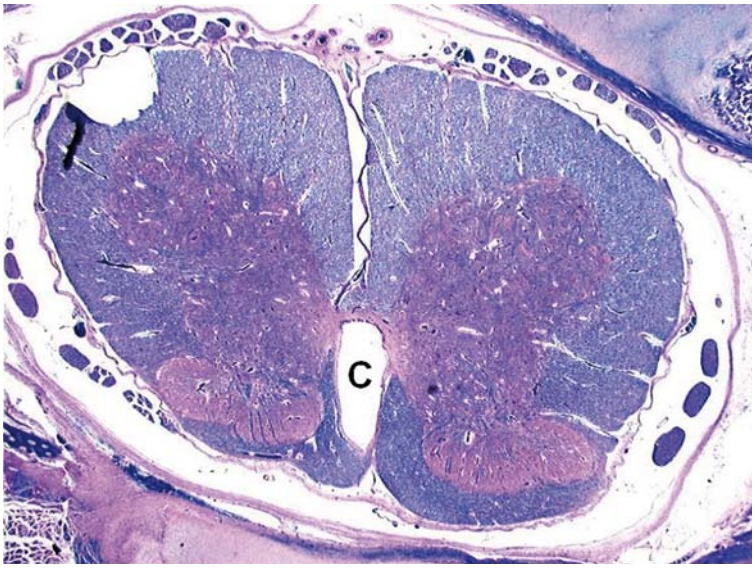

FIG. 3. Cross-section (HE staining) of an uncovered neural tube defect (group 2) showing hydromyelia of the central canal (C). The normal architecture of the spinal cord is largely preserved.

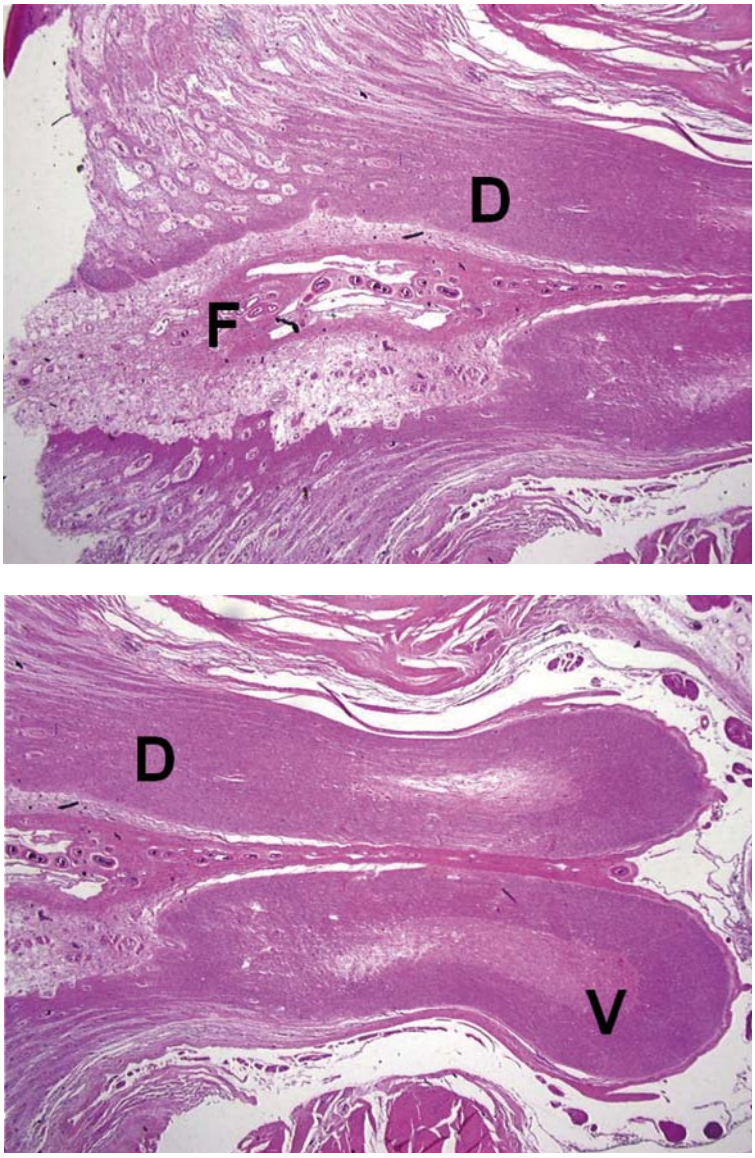

FIG. 4. Cross-sections (HE staining) of an uncovered neural tube defect (group 2) showing central sagittal splitting of the spinal cord with severe damage of the dorsal columns and horns (D). There is widening of the posterior fissure (F), the dorsal horn has been split and has merged with the skin. The architecture of the ventral horn (V) has been better preserved.

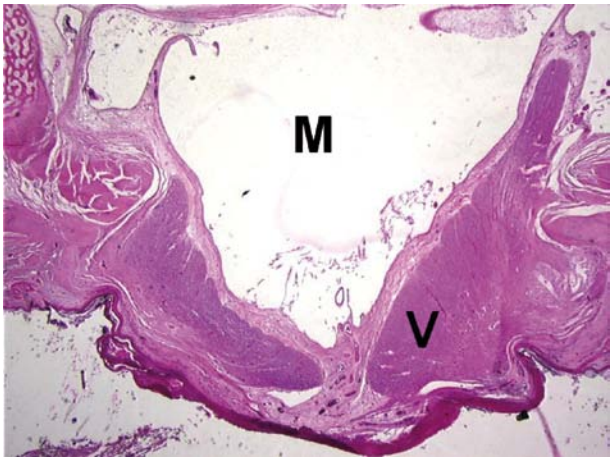

FIG. 5. Cross-section (HE staining) of the dorsal part of the exposed spinal cord of an uncovered neural tube defect (group 2) showing destruction of the normal architecture with flattening of the spinal cord and the formation of a meningomyelocele. V = Ventral horn; M = meningomyelocele.

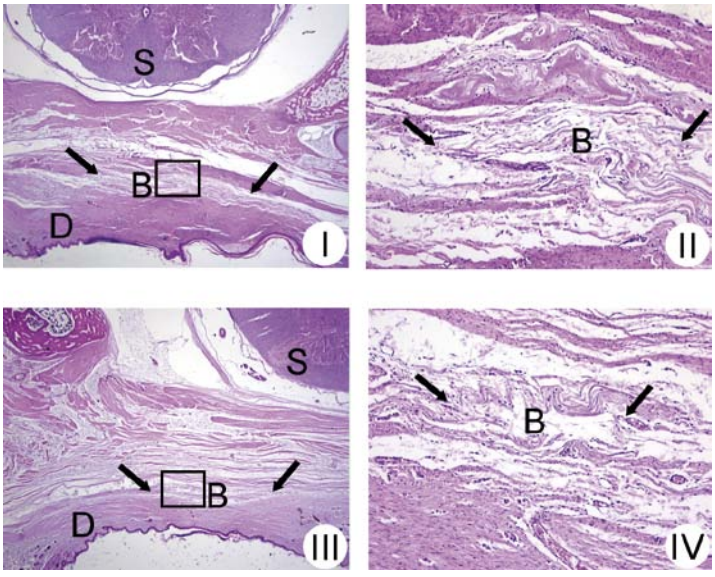

FIG. 6. Cross-sections (HE staining) of the dermis and subcutaneous tissues covering the spinal cord in a lamb with the neural tube defect covered with an UMC biomatrix (I) and an SIS biomatrix (III). Pictures II and IV are detailed views (x 100) of the rectangles in picture I and III (x 12.5), respectively. Remnants of the biomatrices can be found just beneath the dermis in the subcutaneous tissues (between the arrows). Ingrowth of fibroblasts is visible in the detailed views. The scar tissue covering the defect contains no adnexes of the skin. B = Remnants of the biomatrix; D = dermis; S = spinal cord.

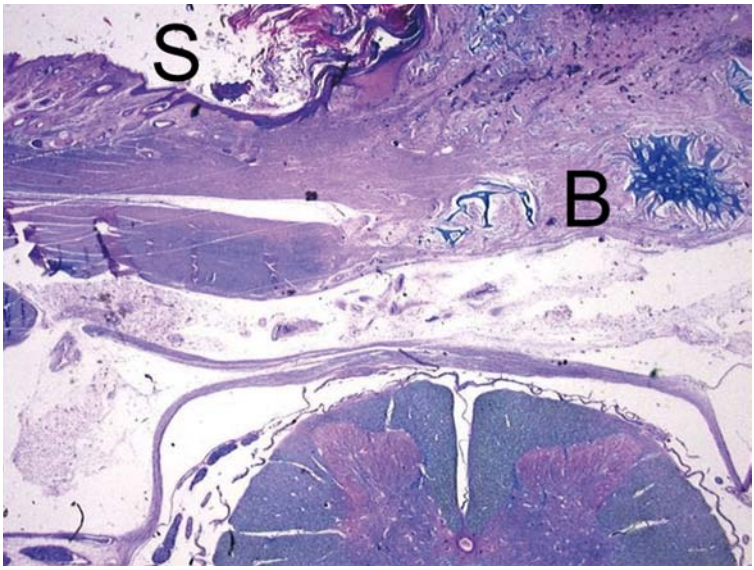

FIG. 2. Cross-section of the skin (S) covering the spinal cord in a lamb with the spina bifida covered with a collagen biomatrix (group 1). The architecture of the spinal cord is preserved. Small remnants of the biomatrix (B) can be found in the subcutaneous tissues. Luxol fastblue-HE. x25.

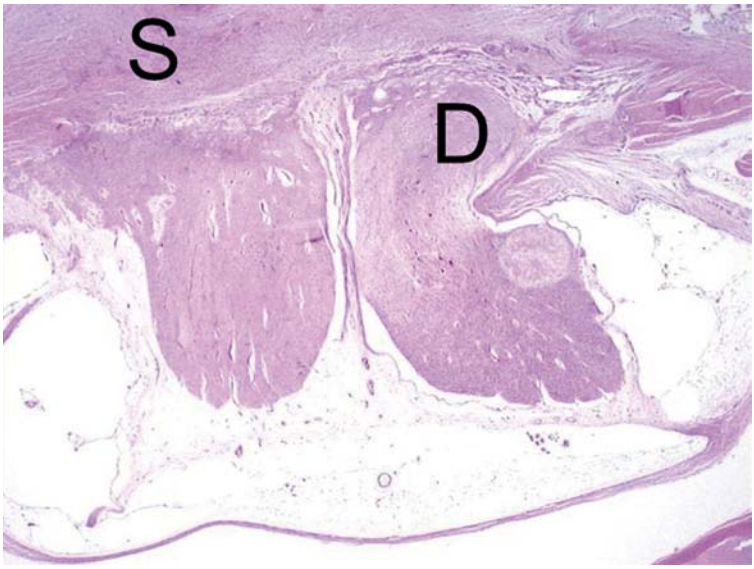

FIG. 3. Cross-section of the spinal cord in a lamb with the spina bifida not covered (group 2). There is a clear abnormal architecture of the neural tissue. The spinal cord is separated and the dorsal horns (D) are merged with the skin (S). HE. x25.

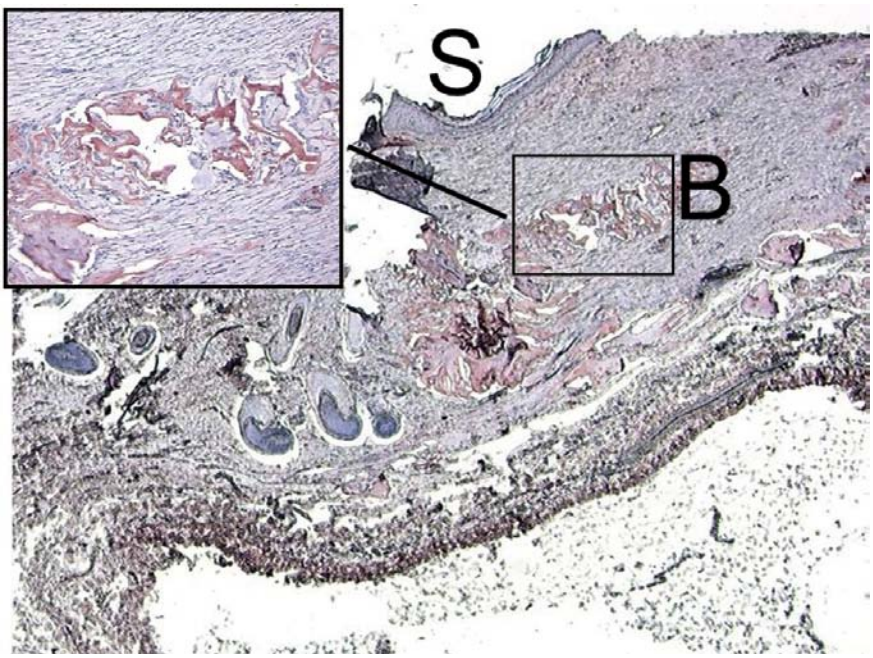

FIG. 4. Cross-section of the dermis and subcutaneous tissues of a sham-operated control lamb (group 3). Remnants of the biomatrix (B) can be found just beneath the skin (S) in the subcutaneous tissues. Ingrowth of fibroblasts is visible in the detailed view. Immunohistochemical staining. x50, insert x200.

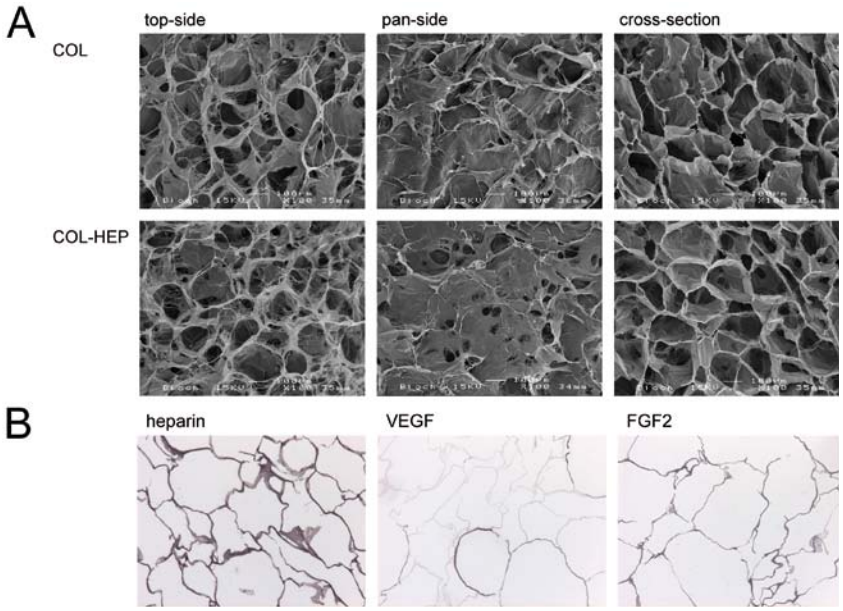

FIG. 1. Scanning electron microscopical images of collagen scaffolds cross-linked in the absence (top row) or presence of heparin (bottom row) (A). Shown are the top view, bottom view and cross-section. The more closed pan side was implanted such that it was in contact with the amniotic fluid. Immunolocalisation of heparin, VEGF and FGF2 in the COL-HEP/VF scaffolds (B). Note that heparin and FGF2 were evenly distributed through the scaffold, while VEGF was mainly found at the edge of the scaffold.

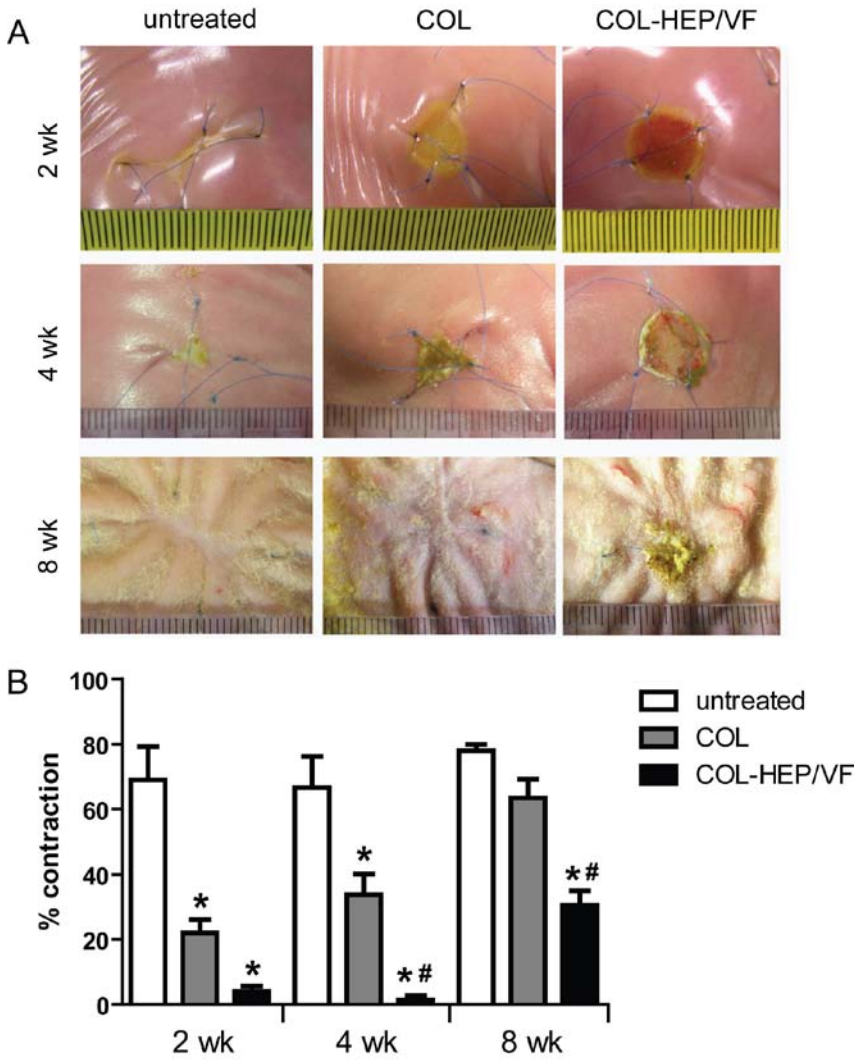

FIG. 2. Macroscopical overview of wound healing after surgery at 93 days' (2 weeks post surgery), 107 days' (4 weeks post surgery) and 140 days' gestation (term) (A). Note the shape of contraction of the untreated lesion and the reddish appearance of the wound treated with COL-HEP/VF. Percentage of wound contraction (B). At 2 and 4 weeks post surgery less contraction was present in COL treated wounds compared to untreated wounds. At all time points less contraction was present in COL-HEP/VF treated wounds compared to untreated wounds. * significant difference between untreated and COL or COL-HEP/VF ($p < 0.05$). # significant difference between COL and COL-HEP/VF treated ($p < 0.05$).

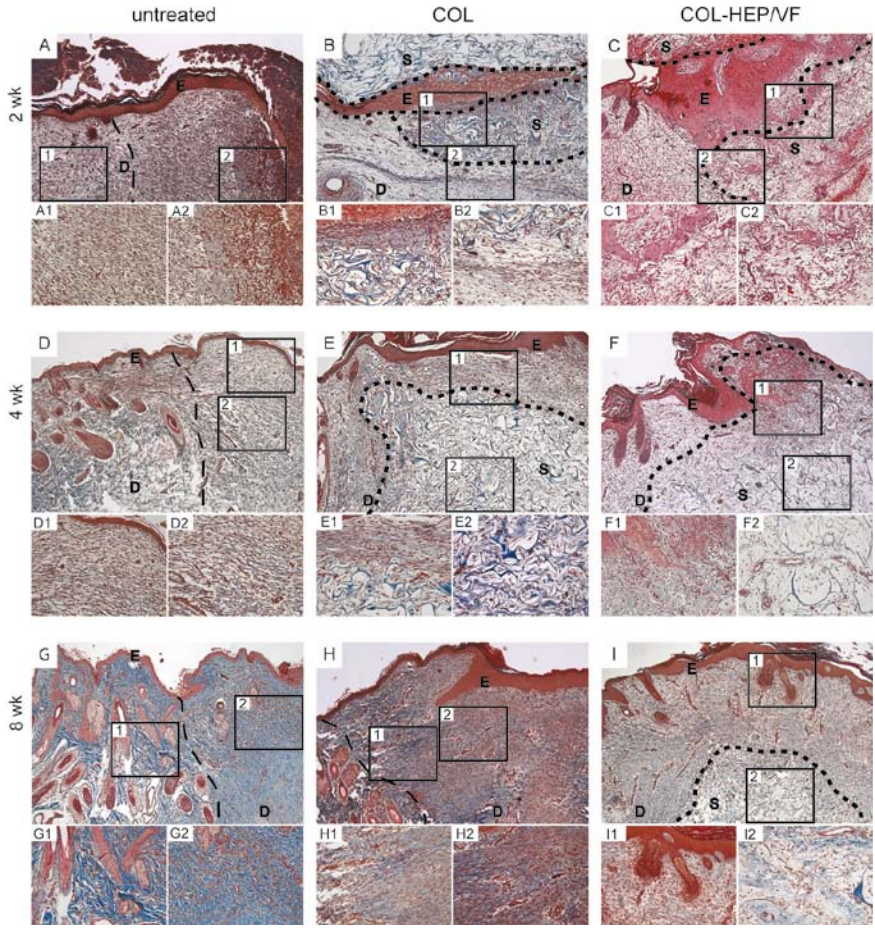

FIG. 3. Masson's trichrome staining of skin defects at different time points. Untreated defect at 2 weeks (A), 4 weeks (D) and 8 weeks (G). Defect treated with COL at 2 weeks (B), 4 weeks (E) and 8 weeks (H). Defect treated with COL-HEP/VF at 2 weeks (C), 4 weeks (F) and 8 weeks (I). The black striped line indicates the edge of the defect; the black dotted line indicates the border between surrounding tissue and the scaffold. E = epidermis, D = dermis, S = scaffold. The boxed areas denote the corresponding region of higher magnification images. Original magnifications: x50 (A–I) and x200 (A1-I1).

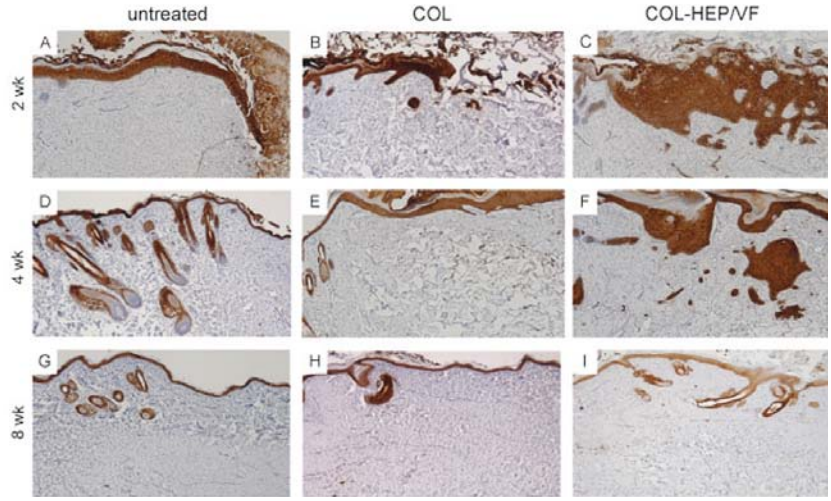

FIG. 4. Epithelialization. Cytokeratin staining of skin defects at different time points. Untreated defect at 2 weeks (A), 4 weeks (D) and 8 weeks (G). Defect treated with COL at 2 weeks (B), 4 weeks (E) and 8 weeks (H). Defect treated with COL-HEP/VF at 2 weeks (C), 4 weeks (F) and 8 weeks (I). Original magnifications: x50 (A–I).

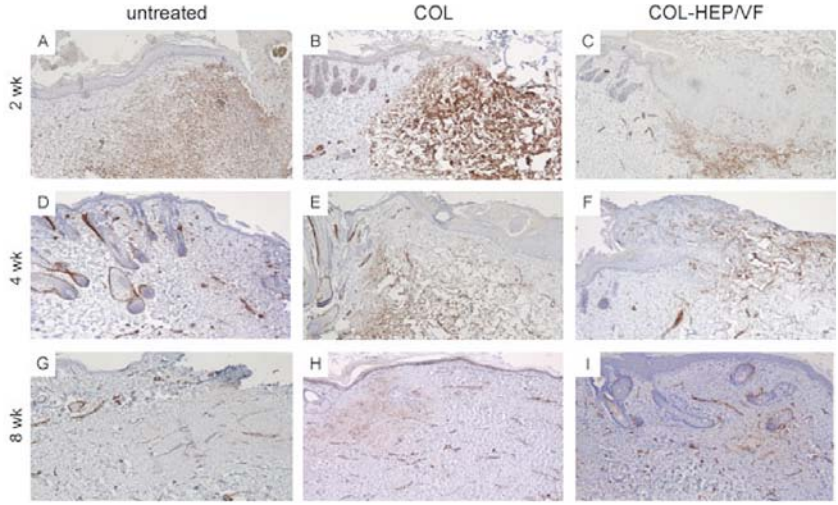

FIG. 5. Myofibroblasts. α-SMA staining of skin defects at different time points. Untreated defect at 2 weeks (A), 4 weeks (D) and 8 weeks (G). Defect treated with COL at 2 weeks (B), 4 weeks (E) and 8 weeks (H). Defect treated with COL-HEP/VF at 2 weeks (C), 4 weeks (F) and 8 weeks (I). Original magnifications: x50 (A–I).

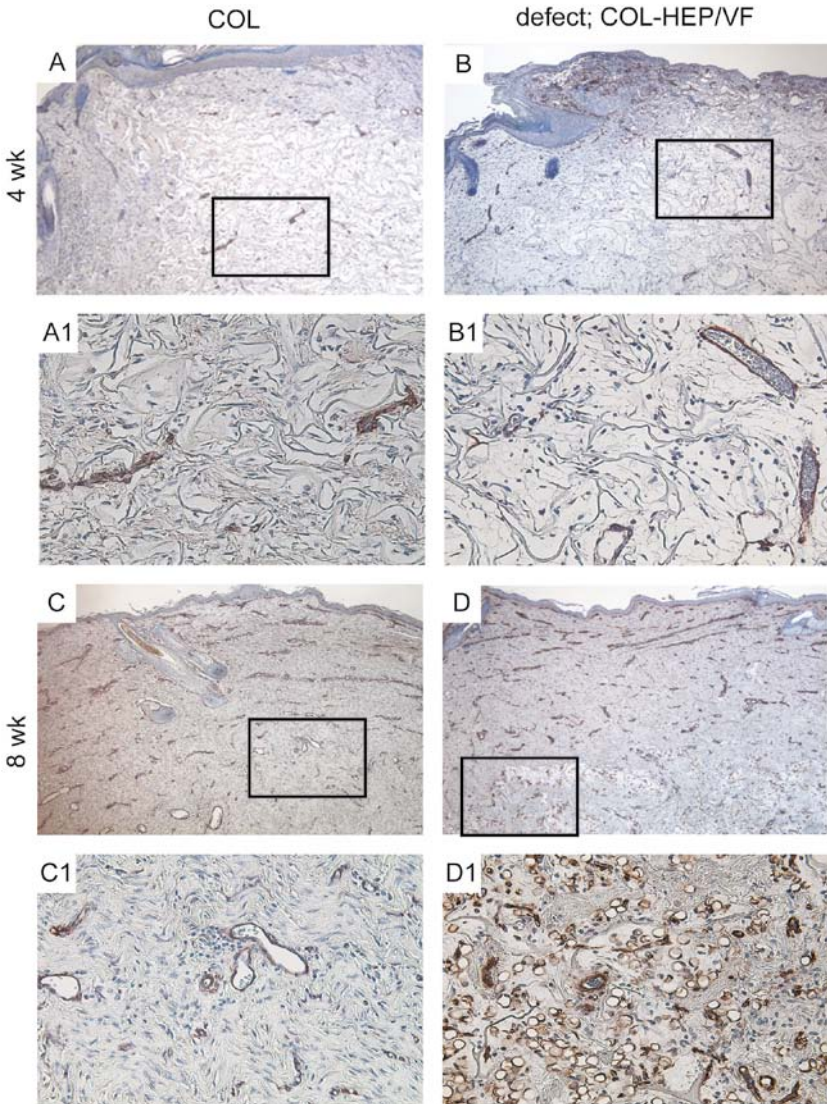

FIG. 6. Vascularization. Collagen IV staining of skin defects at different time points. Defect treated with COL at 4 weeks (A) and 8 weeks (C). Defect treated with COL-HEP/VF at 4 weeks (B) and 8 weeks (D). The boxed areas denote the corresponding region of higher magnification images. Original magnifications: x50 (A–E) and x200 (A1-E1).

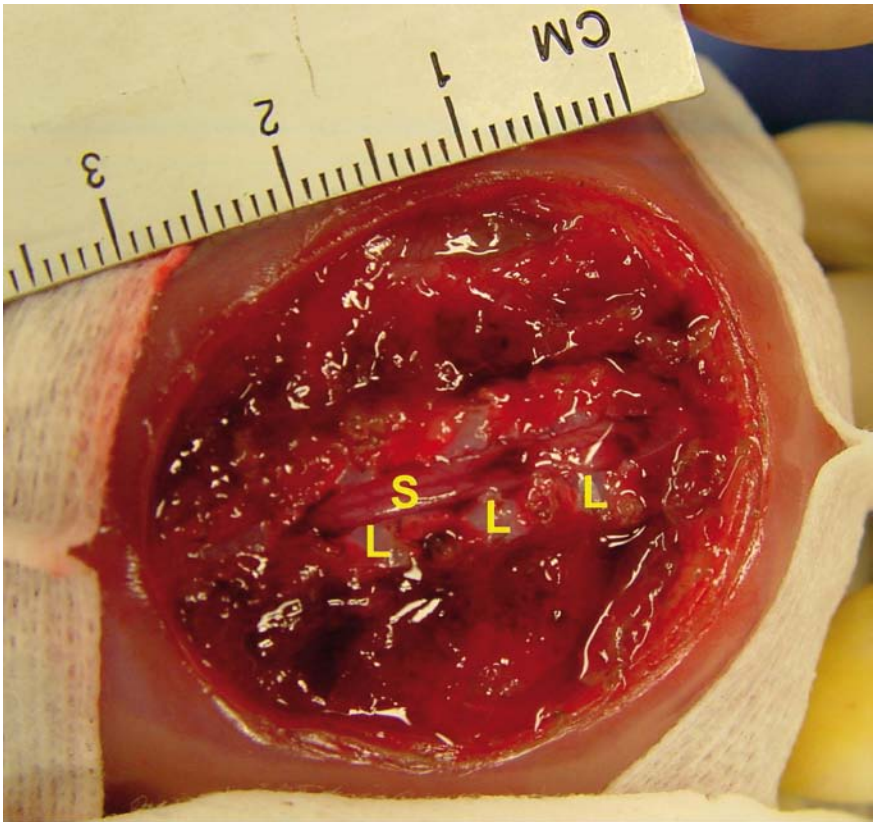

FIG. 1. Surgically created neural tube defect.
Skin, paraspinal muscles and soft tissue has been excised. Laminectomy is performed of 3 lumbar vertebrae (L). The spinal cord is exposed (S).

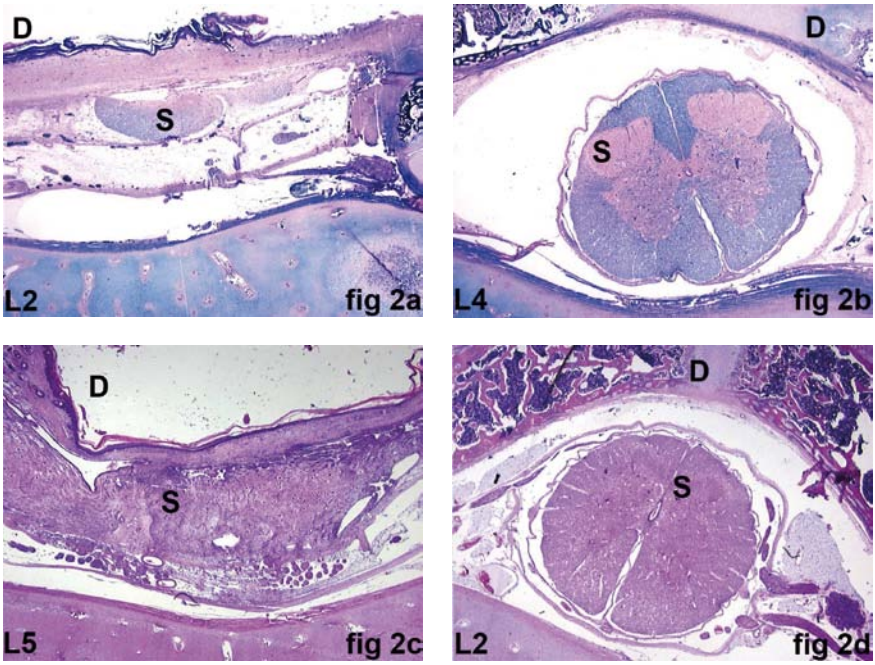

FIG. 2. Cross-sections of the spinal cord in a lamb with a NTD (group 1, fig. 2a and b) and with a NTD and AFE (group 2, fig. 2c and d).
At lumbar level 2 in fig. 2a there is a clear abnormal architecture of the neural tissue; the posterior funiculi and horns are missing. At lumbar level 4 in fig. 2b the spinal anatomy is grossly normal. At lumbar level 5 in fig. 2c, cyst formation in a flattened anterior horn, syringomyelia, hydromyelia and a dilated central canal are seen. The posterior horns are fused with the subcutaneous tissue. At lumbar level 2 the anatomy is normal (D: dorsal side, S: spinal cord). HE-Luxol fast blue (fig. 2a and b), HE (fig. 2c and d). x25.

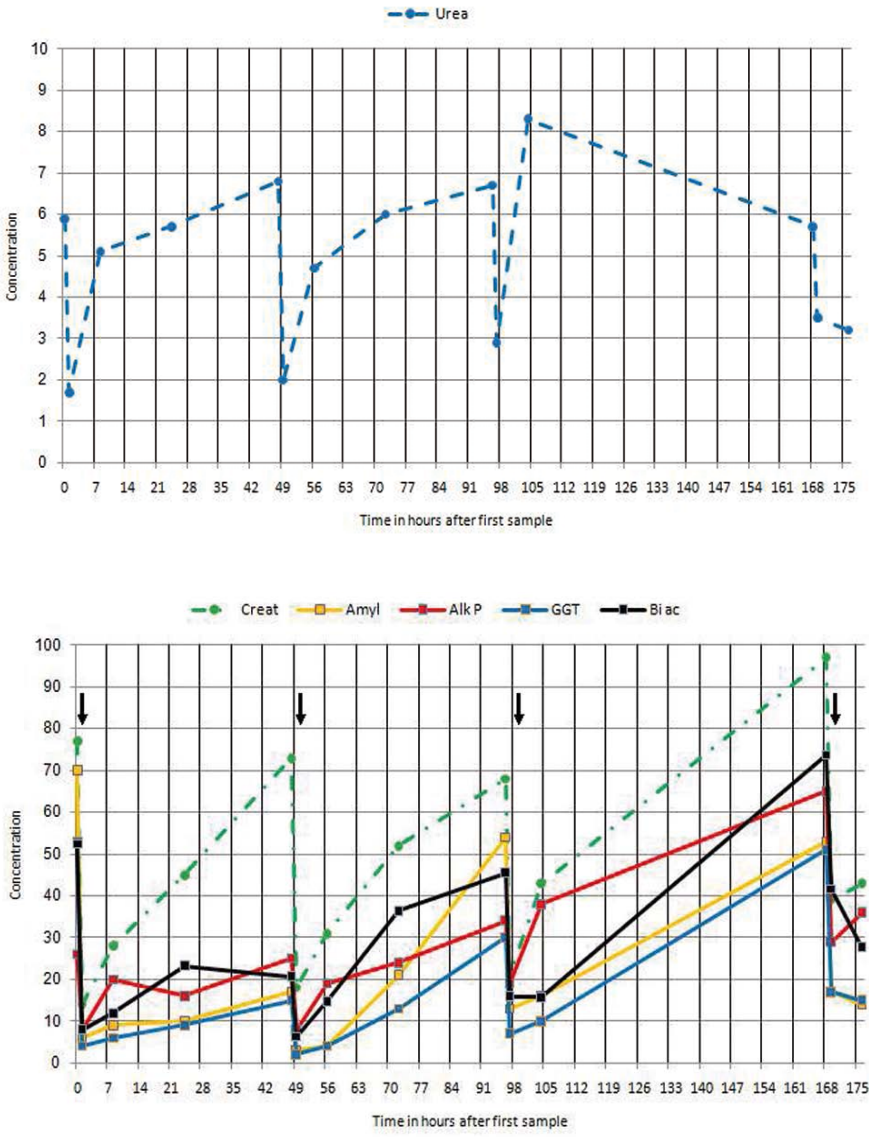

FIG. 3. Concentration of biochemical constituents in AF of urea (top) and creatinine, amylase, alkaline phosphatase, gamma-glutamyltransferase and bile acids (bottom) before and after AFE at 4 time points with 350, 300, 180 and 120 ml warm sterile physiologic saline solution (0.9%) respectively. Y-axis indicates the concentration of waste products (urea in mmol/l, creat: creatinine in μmol/l, amyl: amylase in U/l, alk.P: alkaline phosphatase in U/l, GGT: gamma-glutamyltransferase in U/l, bi ac: bile acids in μmol/l). X-axis indicates the time in hours after first sample. Urinary waste products: dotted lines, gastrointestinal waste products: not interrupted lines. Arrows indicate the first sample 1 hour after AFE.

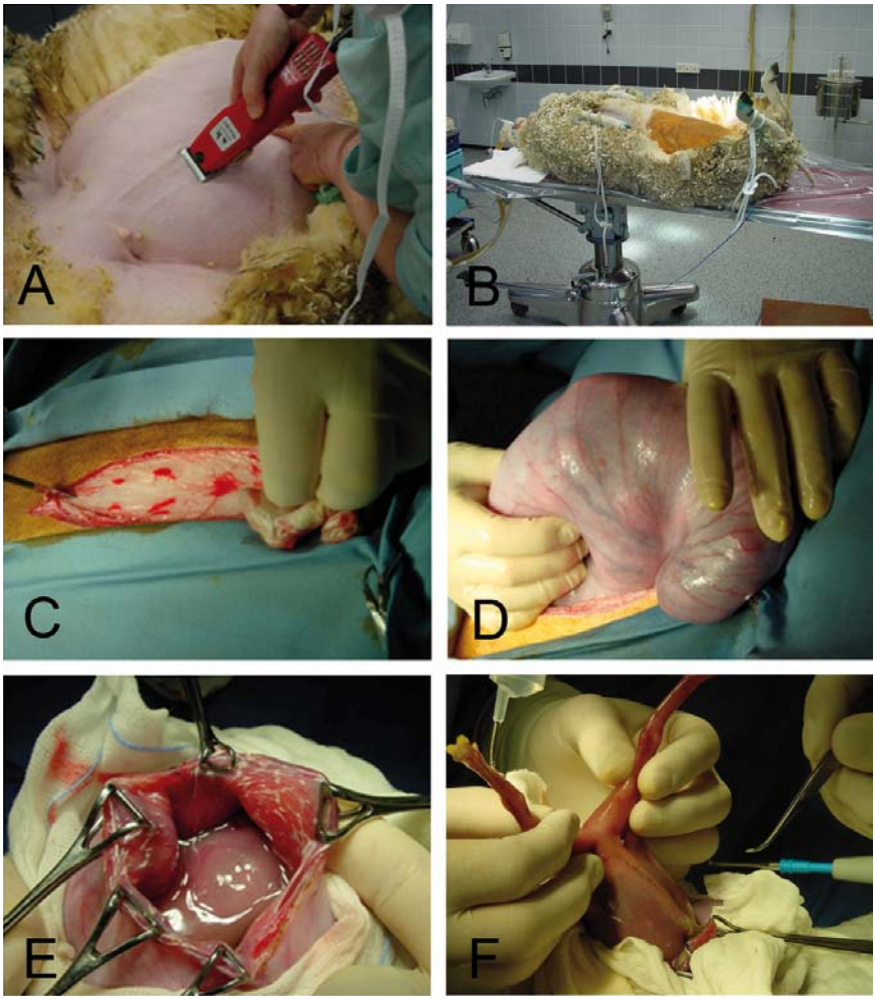

FIG. 1. Overview of intra operative management of the pregnant ewe and the fetal lamb (79 days' gestation). The abdominal wall was shaved, washed with soap, aseptically prepared and sterilely draped before a low midline laparotomy was performed (A, B and C). The appropriate horn of the uterus was exteriorized and a hysterotomy was performed (D and E). The fetus was exposed through the uterine incision (F).

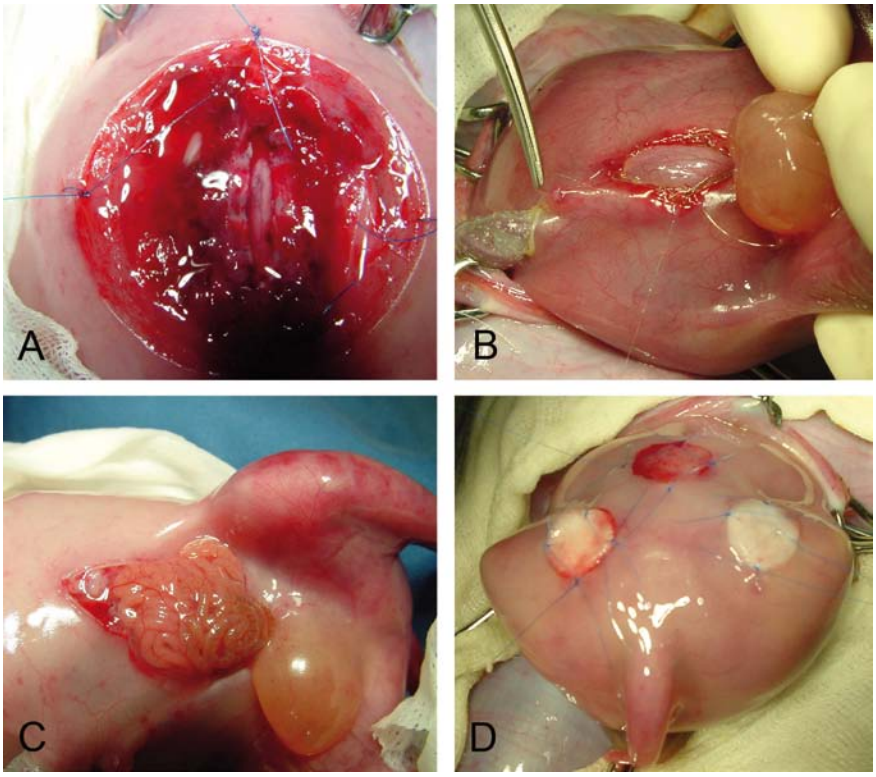

FIG. 2. Overview of different surgical procedures in fetal sheep (79 days' gestation). Fig. 2A. Neural tube defect. After excising skin, paraspinal muscles and soft tissue, a laminectomy was performed and the dura was opened. The exposed spinal cord is visible [16-18]. Fig. 2B. Bladder exstrophy. After opening of the abdominal wall and making an incision in the anterior bladder wall, the edges of the bladder wall were sutured to the abdominal wall resulting in an exstrophic bladder [19]. Fig. 2C. Gastroschisis. After making an incision in the left lower quadrant of the fetal abdominal wall the bowel was exposed through this full-thickness abdominal wall defect [20]. Fig. 2D. Skin defects. Three circular lesions were made on the back of the fetus by excising the skin. One lesion was left uncovered (superior) and two lesions were covered with a collagen scaffold (inferior) [21].

FIG. 3. Images of the refined housing conditions of the operated sheep and lambs at the farm of the Central Animal Facility, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. Separately housed pregnant sheep after fetal operation (A). After normal delivery, sheep are housed with lambs for proper care (B). After 1 to 2 months, sheep and lambs are housed in herds and enjoy the open field (C).