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Complete tissue coverage achieved by scaffold-based tissue engineering in the fetal sheep model of Myelomeningocele

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ABSTRACT

Myelomeningocele (MMC) is the most severe form of spina bifida, one of the most common congenital anomalies. Although open fetal surgical repair of the MMC defect has been shown to result in improved outcomes, a less invasive approach applicable earlier in gestation than the current open surgical approach between 19 and 26 weeks of gestation is desirable for further improvement of neurological symptoms, as well as reduction of maternal and fetal risks. We previously reported the therapeutic potential of a scaffold-based tissue engineering approach in a fetal rat MMC model. The objective of this study was to confirm the long-term efficacy of this approach in the surgically created fetal sheep MMC model. Gelatin-based or gelatin/collagen hybrid sponges were prepared with and without basic fibroblast growth factor (bFGF) incorporation. The defect was covered by a sponge and secured by a supporting sheet with adhesive at 100 days of gestation or the gelatin/collagen hybrid with bFGF was secured with adhesive without the sheet. Although sheets were found detached at term (140 days' gestation), both gelatin-based and gelatin/collagen hybrid sponges had integrated within the newly formed granulation tissue, resulting in complete coverage of the MMC defect. The release of bFGF from sponges resulted in enhanced formation of granulation tissue and epithelialization. There was also evidence of improved preservation of the spinal cord with less associated damage on histological analysis and reversal of hindbrain herniation. These experiments provide important proof-of-principle evidence of the efficacy of scaffold-based tissue engineered coverage for the prenatal treatment of MMC.
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1. Introduction

Myelomeningocele (MMC), a common congenital malformation affecting 1 in 2000 live births, is the most severe form of spinal bifida, and represents the first non-lethal disorder treated by open fetal surgery. MMC is defined as a defect in the closure of the vertebral arches and soft tissues of the back, resulting in exposure of the meninges and neural elements, with devastating neurological consequences including lower extremity paralysis, neurogenic bladder, and hindbrain herniation. A "two-hit" hypothesis has been proposed; the first "hit" is the primary failure of neurulation, and the second "hit" is the chemical and mechanical injury to exposed neural elements that occur throughout the remainder of gestation [1]. The majority of neural damage occurs during the second hit, providing a compelling rationale for prenatal therapy [2–4]. Promising preliminary data in a large animal model [5–7] and preliminary clinical experience [8–10] led to a National Institutes of Health (NIH)-sponsored, multicenter, randomized-controlled clinical trial, the Management of Myelomeningocele Study (MOMS)

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[11], which compared *in utero* surgical repair of the MMC defect against standard postnatal repair. It concluded that fetal surgical closure resulted in significant improvement in rates of shunt placement, degree and presence of hindbrain herniation, as well as functional neurological outcomes, including the ability to walk without orthotics. However, not all fetuses benefited from prenatal repair, and the procedure is relatively invasive for the mother. It is our view, that tissue coverage of the defect earlier in gestation than the current open surgical approach performed between 19 and 26 weeks of gestation, i.e. soon after MMC can be detected at approximately 15–18 weeks, will be required for further improvement in outcomes. As conventional surgical techniques, such as suturing and approximating tissues, are not possible during that period, the ideal repair would utilize tissue engineering techniques. We have hypothesized that the tissue engineered generation of fluid-impermeable tissue coverage of the exposed spinal cord at early gestation would lead to both neural protection and reversal/prevention of the Arnold-Chiari malformation, and could be an alternative to open fetal surgical closure. Our rationale for the use of gelatin for a scaffold was related to its cytocompatibility and controllable degradation, the conformability of gelatin hydrogel to various shapes, and its ability to be a carrier for the controlled release of growth factors with biological activities [12–15]. We have previously confirmed the therapeutic potential of a gelatin-based tissue engineering approach with sustained release of basic fibroblast growth factor (bFGF) in the retinoic acid-induced fetal rat MMC model [16,17]. The latter model, albeit relevant, allows the scaffold to remain in situ for only three days limiting the interpretation of results. In the present study, we used the surgically created sheep model of fetal MMC, to test the long-term therapeutic potential of the scaffold-based tissue engineering approach for prenatal closure of MMC. This model allows fetal observation for up to 40 days following tissue engineering repair, and can provide findings that could be translated to clinical practice. We also compared an additional scaffold material, the gelatin/collagen hybrid sponge, to assess whether the addition of collagen which is cytocompatable and commercially available, provided additional efficacy. We applied the gelatin/collagen hybrid sponge to the MMC defect in two different ways. One was our standard application (sponge and sheet composite)-where the scaffold is cut to the exact size of the MMC defect and secured by coverage with the sheet. The other consisted of application of an oversized gelatin/collagen hybrid sponge alone without an overlying sheet to simplify the approach. This approach avoids the necessity of cutting the sponge to size during surgery and is more amenable to our ultimate goal, of repair of MMC earlier in gestation using only fetoscopic techniques. We evaluated local tissue coverage over the MMC defect and residual spinal cord histologically as well as hindbrain herniation by magnetic resonance imaging (MRI).

2. Materials and methods

2.1. Biomaterials

Gelatin with an isoelectric point (IEP) of 5.0 (molecular weight = 99,000) was prepared through alkaline processing of bovine bone (Nitta Gelatin (Osaka, Japan)). Gelatin/collagen hybrid sponges contained a mixture of 9 parts gelatin isolated from pig dermis with an IEP of 5.0 and a molecular weight of 99,000, and 1 part atelocollagen isolated from pig tendon with an IEP of 8.5 and a molecular weight of 300,000, were kindly provided by Gunze Limited (Kyoto, Japan). b-FGF was kindly supplied by Kaken Pharmaceuticals (Tokyo, Japan) respectively. Glutaraldehyde (GA) and Glycerol were purchased by Sigma–Aldrich (St. Louis, MO, USA) and Nacalai Tesque (Kyoto, Japan) respectively.

2.2. Preparation of scaffolds and incorporation of bFGF

In this study, two sponges were used as scaffolds for tissue regeneration: one gelatin-based and another that was a gelatin/collagen hybrid. Moreover, a gelatin sheet was utilized as a supporting/covering membrane to allow maintenance of adherence of the sponges on MMC defects. Gelatin sponges were prepared with hydrothermal crosslinking [13,16]. Briefly, 3% w/v aqueous gelatin was mixed and casted, and was subsequently frozen at −20 °C, lyophilized, and crosslinked hydrothermally at 160 °C for 72 h. Supplied gelatin/collagen hybrid sponges were prepared with GA crosslinking [18,19]. Briefly, these sponges were created by mixing and casting aqueous 3% w/v gelatin and 0.3% w/v atelocollagen, which were then frozen, lyophilized, and crosslinked with 0.2%w/v GA. All sponges were ethylene oxide sterilized prior to use. Flexible gelatin sheets were prepared with addition of glycerol as the plasticizer in GA crosslinking. Briefly, 5% w/v aqueous gelatin was mixed with 0.5%w/v GA and 5% w/v glycerol, and the mixture was air-dried and UV sterilized.

For loading with bFGF, freeze-dried sponges measuring 3 mm in thickness were gridded into 1 cm squares and 1 μL of 10 μg/μL bFGF was applied at each grid intersection, and allowed to absorb overnight before use, providing approximately 10 μg of bFGF for each cm^2 of the sponge,. For group B (gelatin sponge and sheet composite without bFGF), 1 μL of Normal Saline was applied in an identical fashion. In the animals designated for application of the sponge and sheet composite, the sponge was cut to fit the exact size of the MMC defect at the time of repair and a sheet was prepared in sizes large enough to allow sponge coverage (Fig. 1-C, D). For application of the gelatin/collagen hybrid sponge alone, oversized sponges were cut larger than the MMC defect, allowing the sponge edges to be glued to the surrounding skin (Fig. 1-E).

2.3. Animal experiments

All experimental protocols were approved by the Institutional Animal Care and Use Committee at The Children's Hospital of Philadelphia and followed guidelines set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.4. Experimental groups

A total of five groups were prepared as follows (Table 1): Group A (n = 1): Sham operated control which is untreated MMC; Group B (n = 1): Tissue engineering repair with gelatin sponge and sheet composite without bFGF; Group C (n = 5): Tissue engineering repair with gelatin sponge and sheet composite with bFGF; Group D (n = 1): Tissue engineering repair with gelatin/collagen hybrid sponge and sheet composite with bFGF; Group E (n = 1): Tissue engineering repair with gelatin/collagen hybrid sponge alone with bFGF.

2.5. Creation of MMC in fetal sheep

The procedure for creating MMC defects in fetal sheep was described previously [20]. Briefly, at 72–73 days' gestation, the ewe underwent general anesthesia with isoflurane, the uterus was exteriorized through a laparotomy incision, and the back of the fetus was exposed via a hysterotomy. After resection of a circular section of skin and subcutaneous tissue over the lumbar spine, the posterior spine was exposed by excising paraspinous muscles bilaterally, and a complete laminectomy of lumbar levels L1 to L5 with myelotomy was performed (Fig. 1-A). After returning the fetus into the uterus, amniotic fluid was replaced with normal saline, and the hysterotomy and maternal laparotomy were closed.

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