Repair of oronasal fistulas with human amniotic membrane in minipigs

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Abstract

We evaluated the use of multilayer human amniotic membrane (HAM) as a grafting material for the repair of mid-palate oronasal fistulas in seven Berlin minipigs. After two weeks, three animals had the fistulas repaired with multilayered HAM grafts, three had them repaired with a collagen-based dermal substitute (INTEGRA®, Integra Life Sciences, Plainsboro, NJ, USA), and one fistula was left untreated to serve as a control. Grafts were interposed between the oral and nasal mucosa, traversing the fistulas. After healing for 40 days, the pigs were killed for clinical, histological, and immunohistochemical examination.

Two of the three fistulas closed with HAM were successful, the diameter of the third was reduced in size, and there was no change in the diameter of the fistula in the control.

This study shows successful closure of oronasal fistulas in minipigs using interposed grafts of cryopreserved HAM, and offers promise as a simple and effective technique for tension-free closure of such fistulas.

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Introduction

The formation of an oronasal fistula is a serious complication of the primary repair of cleft palates. Its incidence varies widely, ranging from 0% to 68% in published reports.1 Many causes have been proposed for the formation of a fistula after repair of a cleft palate, including paucity of local tissue for closure, and excessive scarring.1–5 Various techniques for repair have been suggested including local2 and free flaps,3 tissue expansion,4 and the use of allogenic tissues and biomaterials.1,5

Fresh human amniotic membrane (HAM) has been used for nearly a century in reconstructive surgery,6 but its use in developed countries has been limited by the risk of cross-infection. The introduction of a method for glycerol-cryopreservation allowed donors and tissue to be retested after the “infection window”, and HAM transplantation became a regular procedure in ophthalmological operations.7 Ophthalmological studies advocated the use of the tissue for its low immunogenicity, minimal inflammation and scarring, and enhancement of epithelialisation.8 Its success in ophthalmology as an adjunct in wound healing encouraged its use in extraocular, immunogenic tissue. Oral and maxillofacial
surgeons have described its use for gingival wound healing, intraoral lining in vestibuloplasty, and in the prefabrication of flaps. To overcome shortcomings in mechanical stability, Kruse et al. proposed a multilayered application for deep corneal defects. In vivo studies have shown that defects in the abdominal wall can be closed successfully with cryopreserved multilayered HAM. We evaluated the use of multilayered HAM as a grafting material for the repair of oronasal fistulas in minipigs.

Materials and methods

We used seven male six-month-old Berlin minipigs (Fa. Schlesier, Großkarolinenfeld, Germany), weighing 22–31 kg (mean 25.8) each. They were kept according to the international principles of laboratory animal care. Water and food were given freely. The study was approved by the local ethics committee and the local government.

Human placenta was obtained under sterile conditions from planned, uneventful Caesarean sections. All women had given written informed consent. A 5 cm × 5 cm piece was prepared from each placenta and stored according to a fixed protocol. Thirty minutes before operation the HAM was thawed, washed with sterile phosphate buffered saline and secured in place with polyglactin 910 (Vicryl®, Ethicon, Norderstedt, Germany) into five layers before application (Fig. 1).

Induction of anaesthesia, intubation, perioperative monitoring, and analgesia were done as described previously. Pigs were killed 40 days after closure of the oronasal fistulas by T61® (Bayer, Leverkusen, Germany) 1 ml/5 kg body weight intravenously.

We made a full-thickness defect in the mid-hard palate (15 mm in diameter) with a 15 mm stainless steel biopsy punch, and subsequent osteotomy of the hard palate. The nasal septum and edges of the bony palate were then rongeured 2 mm beyond the mucosal edges to allow for re-epithelialisation at the periphery of the defects. Fistulas were allowed to mature for two weeks (Fig. 2).

Three of the seven animals were selected randomly to have the fistulas repaired with grafts of multilayered HAM or a collagen-based dermal substitute (INTEGRA® Regeneration Template, INTEGRA Life Sciences, Plainsboro, NJ, USA). Multilayered HAM was prepared as previously described. INTEGRA® dermal substitute is a bilaminar membrane system consisting of a “dermal” component of porous coprecipitate of bovine tendon type I collagen and shark glycosaminoglycan (chondroitin-6-sulfate), and an “epidermal” silicone layer.

Grafts were interposed between the oral and nasal mucosa traversing the palatal fistulas, and sutured with polyglactin suture (Vicryl® 4-0, Ethicon, Norderstedt, Germany) to the lateral edges of the mucoperiosteal bilateral flap created through “von Langenbeck” incisions. Mucosal edges of the fistula were not closed. One pig was not treated after creation of the defect to serve as a control.

Alginate dental casts of the pigs’ palates were taken at the time the fistulas were made. Protective plates were manufactured individually and fixed with three sinus-lift screws after reconstruction of the fistula.

All wounds were monitored morphologically by photograph on postoperative days 3 and 6 under short sedation. They were cleaned of blood clots and food pellets after removal of the palatal appliance, which was fixed again until it was removed completely at day 9 when the width of the fistulas were measured. On day 40 the pigs were killed and the palates examined. The complete hard palate was processed for microscopic tissue analysis.

Paraffin-embedded specimens were cut in 4 μm sections and stained with haematoxylin and eosin, and Elastica van Gieson to visualise and characterise the tissue architecture.

Fig. 1. Multilayered human amniotic membrane graft before insertion (haematoxylin and eosin, original magnification ×60, scale bar 500 μm; detailed view original magnification ×300, scale bar 50 μm).

Fig. 2. Oronasal fistula 14 days after creation.
Immunohistochemical analysis was done on sections using an automated immunostainer (Ventana Medical Systems Inc., Tucson, Arizona, USA) according to the manufacturer’s instructions, with minor modifications. The antibodies used included factor VIII (Dako, Glostrup, Denmark), β-human chorionic gonadotropin (Dako) and pan-cytokeratin (Dako). Positive controls for all antibodies investigated were used to confirm the adequacy of the staining.

Pictures were taken using the Axioskop 2 plus® (Carl Zeiss Light Microscopy, Göttingen, Germany) connected to an AxioCam HRc® (Carl Zeiss MicroImaging, Göttingen, Germany) at magnifications of 5, 10, and 20 times.

Results

The fistula of the untreated control had epithelialised wound margins, and it had decreased to 13 mm. Two of the three HAM-covered fistulas remained closed with no visual evidence of a remaining fistular tract (Fig. 3). The diameter of the third HAM-covered fistula had been 3 mm on day 9 and 6 mm on day 40. All the fistulas treated with INTEGRA® dehisced and had a significantly larger diameter than those treated with HAM on day 40 ($p = 0.043$) (Table 1). Interestingly, all wounds treated with INTEGRA® that were infected on day 9 had resolved by day 40. No wounds closed using HAM were infected.

Staining with haematoxylin and eosin showed that the closed fistulas had re-epithelialised totally in both the oral and nasal sites (Fig. 4). Staining for factor VIII showed that endothelial cells and ingrowth of vessels were present in the sites grafted with HAM (Fig. 5). Numerous fibroblasts and bundles of matured collagen were seen in the scars when stained by Elastica van Gieson (Fig. 6).

### Table 1

<table>
<thead>
<tr>
<th>Animal</th>
<th>Graft material</th>
<th>Size of fistula</th>
<th>Fistula before closure</th>
<th>After closure</th>
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<tr>
<td></td>
<td></td>
<td>Fistula before</td>
<td>After closure</td>
<td>Day 9 Day 40</td>
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<td></td>
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<td>1 Integra®</td>
<td>16</td>
<td>14</td>
<td>6 11</td>
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<td>2 Integra®</td>
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<td>15</td>
<td>8 12</td>
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<td>3 HAM</td>
<td>15</td>
<td>13</td>
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<tr>
<td>4 Integra®</td>
<td>17</td>
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<td>10 12</td>
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<td>5 HAM</td>
<td>16</td>
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<tr>
<td>6 HAM</td>
<td>15</td>
<td>14</td>
<td>0 0</td>
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<tr>
<td>7 Control (no closure)</td>
<td>15</td>
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<td>13 13</td>
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Fig. 3. Postoperative view 40 days after closure with human amniotic membrane. Complete healing was seen.

Fig. 4. Overview on the oronasal complex 40 days after reconstruction of the fistula with human amniotic membrane. Section with palatal and nasal surfaces indicates complete closure (haematoxylin and eosin, original magnification $\times 1.5$, scale bar 4 cm).

Fig. 5. Staining with factor VIII antibody shows dispersed immunoreactions indicating endothelial structures (original magnification $\times 5$, scale bar 1000 $\mu$m).
Fig. 6. Staining with Elastica van Gieson shows sufficient epithelial closure of the oral surface with regularly arranged epithelial cells (original magnification ×5, scale bar 1000 μm).

Fig. 7. No stain with β-human chorionic gonadotropin antibody suggests complete resorption of the fetal tissue graft (original magnification ×5, scale bar 1000 μm).

gonadotropin did not stain, which suggested complete degradation and remodelling of the HAM grafts (Fig. 7).

The three fistulas that had been closed using INTEGRA® and had dehisced were characterised by a pronounced invasion of lymphocytes.

Discussion

Formation of scars and a shortage of tissue complicate the repair of oronasal fistulas. Current methods for their closure can be broadly divided into four groups that use local mucoperiostal flaps, pedicled flaps such as from the tongue, free flaps such as from the forearm, and biomaterials. Donor site morbidity can be substantial so biomaterials seem ideal, but until now none have fulfilled the complex requirements of biocompatibility and integration.

HAM and its extracellular matrix contain components such as growth factors and antimicrobial peptides that suggest that it is an excellent candidate for use as a native scaffold. Unlike other scaffolds it is cheap, and is easy to obtain and to process.

This study shows that oronasal fistulas in pigs can be closed successfully using interposed grafts of cryopreserved multilayered HAM. The intraoral use of fresh monolayered HAM has been reported for mandibular vestibuloplasty, but it is not resilient and was the source of leakage in reconstructed bladders in rats that led to a mortality rate of 42%. Mermet et al. described a limited benefit of monolayered membranes for the treatment of leg ulcers because of rapid degradation. These problems were solved in our earlier study by the successful application of multilayered HAM to repair defects in the abdominal wall in rats.

The clinical course of fistulas reconstructed using HAM was better than that of the control that was closed using INTEGRA®. Unlike INTEGRA®, which has been proposed as an implant for palatal wound healing, the pigs treated with HAM healed uneventfully. Two of three pigs had fistulas closed successfully, and there was a considerable reduction in the diameter of the fistula in the remaining pig 40 days after operation. Histologically, the closed fistulas consisted of matured tissue with collagen bundles and had no remnants of amnion or an appreciable number of lymphocytes. All animals in the INTEGRA® group had inflammation and larger fistulas. Ophof et al. reported complete re-epithelialisation and integration of tissue four weeks after reconstruction of palatal full-thickness wounds in beagle dogs with INTEGRA®, but later studies from this group showed that it did not improve the healing of palatal wounds as revascularisation was too slow to allow survival and integration of INTEGRA® so they dispensed with its use for palatal reconstruction.

Kirschner et al. initiated this study’s pig model for the closure of oronasal fistulas. They used an acellular dermal matrix (AlloDerm®, LifeCell Corporation, New Jersey, USA) to repair fistulas, and achieved excellent results in piglets and patients. Smoothness and pliability were similar to that of oral mucosa, but we used HAM as it is cheaper and widely available, although it is more vulnerable than acellular dermal matrices. Fabrication of a protective plate was essential to avoid mechanical alteration and inflammation that might be caused by food on the palatal folds.

HAM is currently used for reconstruction of the ocular surface and abdominal wall, for bladder repair, treatment of leg ulcers, and burns. It is a useful reconstructive option for the repair of oronasal fistulas and as a surgical patch. Its use in the repair of cleft palates offers new opportunities for its application. With the implementation of prenatal diagnosis it will be possible to harvest and store the amniotic membrane of a child with a cleft as an exclusive tissue source for later reconstruction.

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References