**New concepts of reconstructive techniques with human amniotic
membrane in pelvic floor surgery**

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Ph.D. Thesis

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2018

TABLE OF CONTENTS

List of full papers related to the subject of the thesis	4
List of abstracts related to the subject	5
List of abbreviations	6
1. SUMMARY	7
2. INTRODUCTION.....	9
2.1. Reconstruction of the urinary tract with native tissues	9
2.2. Tissue engineering in urology	9
2.3. Amniotic tissue.....	11
2.4. The IDEAL recommendations	13
3. MAIN GOALS	14
4. MATERIALS AND METHODS	15
4.1. Amnion preparation.....	15
4.2. <i>In vitro</i> amnion studies	17
4.3. <i>In vivo</i> experiments	17
4.3.1. Animals	17
4.3.2. Amnion graft implantation in the bladder and colon.....	17
4.3.3. Explantation and macroscopic studies	18
4.3.4. Histological and immunohistological studies	19
4.3.5. Statistics	20
4.4. Clinical case studies	20
4.4.1. A vesico-vaginal fistula	20
4.4.2. An entero-cutaneous fistula	21
5. RESULTS.....	22
5.1. <i>In vitro</i> experiments	22
5.2. <i>In vivo</i> amnion xenograft bladder reconstruction.....	23
5.2.1. Clinical course and functional results.....	23
5.2.2. Macroscopic examination	23
5.2.3. Microscopic examination.....	24
5.3. <i>In vivo</i> amnion xenograft colon reconstruction	25
5.3.1. Clinical course and functional results.....	25
5.3.2. Macroscopic examination	26
5.3.3. Microscopic examination.....	27
5.4. Graft rejection	29

5.5. Clinical cases	29
5.5.1. A vesico-vaginal fistula	29
5.5.2. An entero-cutaneous fistula	30
6. DISCUSSION	31
6.1. Amnion preparation and <i>in vitro</i> studies	31
6.2. Urinary tract reconstruction with amniotic tissue	33
6.3. Bowel tract reconstruction with amniotic tissue	34
6.4. Limitations of the xenograft animal model	36
6.5. HAM-assisted repair of a vesico-vaginal fistula	37
6.6. HAM dressing for the treatment of chronic wounds.....	38
6.7. The project with regard to the IDEAL recommendations.....	39
7. SUMMARY OF THE NEW FINDINGS.....	41
8. ACKNOWLEDGEMENTS	42
9. LIST OF REFERENCES	44

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List of abbreviations

BAM	bladder acellular matrix
DAB	diaminobenzidine
DFU	diabetic foot ulcer
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial–mesenchymal transition
FBR	foreign body reaction
FDA	Food and Drug Administration
HAM	human amniotic membrane
H&E	haematoxylin and eosin
HGF	hepatocyte growth factor
IDEAL	Innovation, Development, Exploration, Assessment and Long-term Study
IDEAL-D	IDEAL-Device
KGF	keratinocyte growth factor
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
PEI	Paul Ehrlich Institute
PBS	phosphate-buffered saline
SIS	small intestinal submucosa
TE	tissue engineering
TGF- β	transforming growth factor β
VVF	vesico-vaginal fistula

1. SUMMARY

Due to the aging of society and the growing number of oncology cases, there is a need for new and efficient reconstruction techniques in urology. Native tissue is often not available or can be in bad condition after multimodal treatments with surgery and radio-chemotherapy. Various methods and materials have been developed to date to replace the damaged tissues. The possible indications embrace the partial or complete replacement of the ureter, bladder or urethra, repair of complex fistulas and protection of anastomoses. One of the strongest tissues in the human body and one with multiple growth factors, human amniotic membrane (HAM) could serve as a potential graft in reconstructive urology. Amniotic tissue can be easily harvested during Caesarean sections and is therefore broadly available. However, by now its application is limited to cornea replacement and burn therapy. Therefore, our first aim was to develop a manufacturer-independent, standardized *in vitro* process for the preparation of amnion grafts. In this line, we planned to develop a sterilization and storage technique for frozen and dried HAM with a preserved extracellular matrix. As a next step, we employed an *in vivo* xenologous model to test previously constructed multilayer HAM grafts and to investigate the capacity to repair small defects in the rat bladder and intestinal tract. HAM provided a durable graft with good functional and histological results for bladder grafting. The initial inflammatory reaction decreased, with no signs of degradation observed over six weeks of follow-up and only subtle signs of graft rejection detected. However, HAM was not suitable for intestinal reconstruction due to an increased rate of adhesions. Next, by following the steps in the Innovation, Development, Exploration, Assessment and Long-term Study for Devices (IDEAL-D) system of surgical innovation, we defined possible human indications. The first-in-human stage of innovation involved clinical cases of complex fistulas, a vesico-vaginal fistula and a chronic wound with an entero-cutaneous fistula, which were treated successfully with HAM allografts. Based on the promising clinical data, further directions could be planned, targeting ureteral reconstruction or the protection of urethra anastomoses.

ÖSSZEFOGLALÁS

Az öregedő társadalom és az onkológiai esetek növekvő száma miatt új és hatékony rekonstrukciós technikákra van szükség az urológiában is. Autológ, saját szövet nem áll mindig rendelkezésére, vagy a korábbi sebészi beavatkozások és radio-kemoterápiás eljárásokat követően az állapotuk nem kielégítő. Az ureter, a húgyhólyag és a húgycső részleges vagy teljes pótlására, a komplex fistulák megoldására, az anasztomózisok védelmére, a sérült szövetek helyettesítésére többféle módszer és lehetőség került már kidolgozásra. A humán amnion membrán (HAM) az emberi test egyik legerősebb szövete, növekedési faktorokat tartalmaz, császármetszések során viszonylag könnyen, széles körben beszerezhető, de a klinikai alkalmazása mindeddig főképp a szaruhártya cseréjére és az égés terápiájára szorítkozott. Feltételeztük, hogy a HAM potenciális graftként szolgálhat a rekonstruktív urológiában. Elsődleges célunk az volt, hogy egy gyártó-független, standardizált in vitro eljárást dolgozzunk ki HAM graftok előállítására. E folyamat részeként fagyasztott és szárított, konzervált extracelluláris mátrixszal rendelkező HAM graftok sterilizálási és tárolási technikáját dolgoztuk ki. Következő lépésként xenológ kísérletes modellben vizsgáltuk a többretegű HAM graftok tulajdonságait in vivo körülmények között. Célkitűzésünk szerint megvizsgáltuk a HAM alkalmazásának lehetőségét a húgyhólyag és béltraktus kisebb sérüléseinek megoldására patkányokon. Funkcionális és szövettani eredményeink szerint a HAM graftok tartós, jó megoldást jelentenek hólyagsérülések esetén. A kezdeti gyulladásos reakció idővel mérséklődött, degradációs jelek nem voltak megfigyelhetők, és a graft-kilökődés enyhe jelei jelentkeztek a 6 hetes utánkövetési időszak alatt. Ugyanakkor a HAM alkalmazása nem bizonyult megfelelőnek a bélfal rekonstrukciójára, főképp a fokozott adhézis reakció miatt. Az IDEAL-D (*Innovation, Development, Exploration, Assessment and Long-term Study for Devices*) rendszer előírási lépései szerint haladva meghatároztuk a HAM alkalmazás esetleges humán indikációit. Az innováció első humán fázisát a komplex fistulák, közöttük a vesico-vaginalis fistulák és az entero-cután fistulákkal járó krónikus sebek klinikai esetei jelentették, melyeket sikeresen kezeltünk HAM allograftokkal. Az ígéretes klinikai adatok alapján további rekonstruktív irányok is tervezhetők az urológiában.

2. INTRODUCTION

2.1 Reconstructing the urinary tract with native tissues

Reconstructive and functional surgery has gained increasing importance in urology today due to increased life expectancy and optimized medical treatments. Furthermore, additional interventions with increased risk for higher complication rates are often required in such surgical situations. For example, ileal segments are used for urinary diversion after cystectomy and ureter replacement [Butcher, 1962]. In the case of the neobladder, a 60–70 cm length of ileum segment is selected and incised to prepare a special pouch. One side of the segment is connected to the ureters; the other is connected to the urethra. This approach, however, has many drawbacks, such as the risk for anastomotic strictures, incontinence, increased mucus production, infection rate, stone production, and electrolyte and acid imbalance with resulting nephropathies and secondary malignancies. Other autologous materials, such as buccal mucosa, peritoneum or veins, are also used for reconstruction, but all these techniques and tissues are associated with a series of similar complications and the necessity to extend the surgical procedure, which also increases the operation time and postoperative morbidity.

The Cochrane systematic review database lists only five randomized and quasi-randomized trials (with 355 patients) involving the transposition of an intestinal segment in the urinary tract [Cody, 2012]. The overall level of evidence is low with only IDEAL Stage 0–2b studies (*see explanation later*). The successful intestinal replacement of the ureter dates back more than 50 years [Ghoneim, 2005; Peeker, 2009]; however, the level of evidence is low again, mainly series of case reports in IDEAL Stage 0–1 studies [Chung, 2006; Peeker, 2009]. Nevertheless, it has been demonstrated that the overall complication rate of native tissue reconstruction should be reduced. Collectively, this also suggests that there is a clear need for novel, innovative approaches, and new treatment possibilities should be identified in this field.

2.2. Tissue engineering in urology

The aim of tissue engineering (TE) reconstruction is to improve the postoperative functional outcome using customized natural materials to support the regenerative aspect of the healing process and to avoid or minimize the need for additional surgery. Three key factors are identified for an optimal biological material in urology: proper scaffold construction along with proper regeneration of both the urothelium and

smooth muscle layers [Adamowicz, 2016]. The engineered scaffold aims to replace the extracellular matrix (ECM) and supports cell growth and expansion. Moreover, it should have mechanical and functional structure to replace and incorporate the native tissue. The matrix must provide the cells with a scaffold until they have grown into the environment and at the same time ensure a sufficient supply of nutrients to the cells through the matrix [Atala, *Cur Opin Urol* 2006]. For long-term success, it is important that the scaffold should be either resorbable or autologous to prevent foreign body reactions (FBR) that could lead to scarring [Atala, 2012].

The ECM plays an important role in regenerating the bladder wall and regulating inflammatory reactions in the urinary tract. Collagen and elastin form the mechanical scaffold, while the biochemical and biophysical signals of the ECM are involved in regulating cellular activities, such as adhesion, migration, proliferation, differentiation and functionality [Voytik-Harbin, 2001].

Three different types of tissue matrices are distinguished: (1) synthetic polymers, (2) natural materials, and (3) decellularized matrices [Atala, 2012]. Synthetic polymers present a risk of strong FBR, which can lead to severe complications in the urinary tract [Otto, 2015]. A large number of natural matrices have been investigated in recent years with regard to their applicability for TE, for example, veins, collagen, small intestinal submucosa (SIS) and the bladder acellular matrix (BAM) [Brito-Juarez, 2007; De Filippo, 2015; Mitsui, 2012; Smith, 2002]. Veins have a consistency and stability similar to those of the ureter and can be used autologously, as is known from bypass surgery. They are easy to suture and are waterproof. This led to the idea of using urothelial cells seeded on venous matrices for ureteral replacement [Brito-Juarez, 2007]. Moreover, it was demonstrated that these cells survive for up to one year in contact with urine in pigs. However, an increasing rate of hydronephrosis demonstrated the failure of these matrices after three months or longer periods after implantation [Engel, 2014].

Composite scaffolds were also fabricated by binding collagen with synthetic polymers. Most of the studies with these materials are limited to small animal experiments and case studies with short-term follow-up (IDEAL Stages 0–1).

Advances in tissue engineering technology have enabled seeding of scaffolds with autologous bladder epithelial and smooth muscle cells. For instance, improved bladder capacity was observed in seven patients with myelomeningocele following cystoplasty with engineered bladder tissue [Atala, *Lancet* 2006]. A prospective multi-centre

phase II trial in the United States used a similar cell-seeded biodegradable collagen scaffold for bladder augmentation in children and adolescents with spina bifida (n=11), with follow-up for 36 months; however, improved bladder capacity or bladder compliance was not observed, while several serious adverse events occurred [**Joseph, 2014**].

Another strategy is to apply naturally derived acellular matrices, such as SIS or BAM, in an attempt to induce a native cell seeding of urothelial and smooth muscle cells from the neighbouring native bladder tissue or ureters. SIS was approved by the FDA in 2004, but only a few published results are available to date. The original preclinical investigations led to inconsistent outcomes, primarily because of differences in SIS preparation [**Sievert, 2017**]. Several experimental trials with rat, dog, sheep and porcine models have reported promising short-term results for BAM over the last two decades. Correct muscle alignment, proper innervation and vascularization are crucial for the development of larger contractile tissues that allow physiological voiding. However, the main problems of fibrosis and graft shrinkage of larger scaffolds are present, along with rapid degradation, the need for immune therapy and insufficient cell seeding due to a lack of tissue growth factors [**Mitsui, 2012; Roelofs, 2016; Wefer, 2001**].

The ideal matrix has not yet been clarified, and it remains questionable whether there is a matrix that is suitable for the reconstruction of the entire urinary tract [**Atala, Curr Opin Urol 2006, 2012**]. Another question we should ask is why we have not seen more TE materials adopted in clinical practice. The possible answer is that the main problem is the long transfer of preclinical knowledge to the bedside, starting with cell culture and *in vitro* studies and continuing with *in vivo* animal models, clinical trials and commercialization, with regulatory oversight at all stages [**Ram-Liebig, 2015**].

2.3 Amniotic tissue

The amnion is the innermost layer of the embryo, dividing it from the maternal chorion. The surface of the amniotic membrane is covered by a single layer of cuboidal epithelial cells. The broad and thick basal membrane (150-200 nm) divides the epithelial cells from the avascular, extracellular matrix with mesenchymal stem cells (**Figure 1**). The ECM is characterized by a high proportion of collagen Type IV, V and VII fibres, laminin-1 and laminin-5, and fibronectin [**Resch, 2006**]. The composition of the amniotic ECM is similar to that of the basement membrane of urinary tracts. With a diameter of about 40 µm, the amnion is one of the thickest and

strongest membranes in the human body [Resch, 2006]. There are several key factors that make HAM a potential scaffold for tissue engineering. These are the immune tolerance, promotion of epithelialization, anti-inflammatory, antiangiogenic, antifibroblastic and antimicrobial characteristics. During pregnancy, the immune-privileged amniotic tissue protects the embryo from rejection. HAMs appear to have no antigenic effect, as evidenced by an experiment in humans showing no acute transplant rejection of subcutaneously implanted allograft HAM [Akle, 1981]. HAM contains multiple soluble growth factors that promote epithelial wound healing and limit the inflammatory reaction [Koizumi, 2000]. HAMs secrete the glycoprotein lumican and growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF), which stimulate epithelial cell growth. Epithelial differentiation and migration are promoted, and apoptosis is suppressed [Koizumi, 2000; Meller, 1999]. The stromal matrix of the amniotic membrane in particular has a considerable influence on this antifibrotic development [Li, 2008]. The antibacterial quality of HAM stems from enzymes such as elastase. After introducing a method of cryopreservation in the 1990s, which allowed long-time storage and testing, a versatile use of HAM was started in ophthalmologic surgery [Kim, 1995]. Today, HAM allografts are considered the standard therapy to reconstruct the eye surface as shown in several randomized and controlled trials [Abdulhalim, 2015; de Farias, 2016]. The effectiveness of HAMs has been demonstrated for several other indications, for instance, as skin graft donor site dressing in burn patients and to reconstruct dental defects and oro-pharyngeal fistulas [Kumar, 2015; Loeffelbein, 2014; Rohleder, 2013; Salehi, 2015]. Treatment of young women with cervical and vaginal agenesis with vaginoplasty and HAM grafts resulted in excellent epithelialization and patency of the cervix and vagina [Mhaskar, 2005]. Furthermore, tissue-engineered HAMs have been used as a matrix for cell seeding and expansion of epithelial progenitor cells in ophthalmology, orthopaedics, healing of liver dysfunction, etc. [Tsai, 2000; Yuan, 2015]. Currently, there is a growing interest in expanding the application possibilities of HAMs due to their wide availability, low cost and interesting *in vivo* properties.

Figure 1. Histological cross-section showing the structure of amnion and chorion [Magatti, 2016].

2.4 The IDEAL recommendations

One of the reasons for the missing clinical application of TE is the lack of standardized guidelines. The introduction and description of new surgical methods, innovations or variations does not yet follow clear standardized paradigms as is the case with the development process of new pharmaceutical agents and drugs. It is likely that a consensus is needed for key outcomes (e.g. efficacy, scope and severity of complications, quality of life, etc.) as well as contextual factors (e.g. grading of patient risk factors, severity of comorbid pathology or general health) and between specialist communities, specialties and scientific journals as well in order to standardize reporting. **Innovation, Development, Exploration, Assessment and Long-term Study (IDEAL)** is a new reporting approach, introduced in 2009 by an international panel of surgeons, researchers, journal editors, methodologists, statisticians and other stakeholders who are committed to producing, disseminating and evaluating quality research in surgery [McCulloch, 2009]. Recently, IDEAL stages were adopted to evaluate and regulate the use of medical devices (IDEAL-D) as well (**Table 1**) [Sedrakyan, 2016]. IDEAL provides clear stages for surgical innovation which make it possible to assign all research to its particular level of development and evidence [Sedrakyan, 2016]. This approach is unique and paves a way for an efficient, actionable and transparent quality improvement system for the life cycle of development and post-market monitoring of new medical devices and implants.

Table 1. Stages of the IDEAL-D framework [Sedrakyan, 2016].

		Primary outcome	Study design	Patients
Stage 0	Preclinical	Concept, safety	Experimental studies (animal, cadaver)	
Stage 1 Idea	“First in human”	Innovation	Case report, case series, registration	Single to few (<10)
Stage 2 Development and Exploration	“Tinkering” with device, few adopters	Development, safety, efficacy	Prospective cohort trials	10-100
Stage 3 Assessment	Stable procedure	Compare to standard, clinical outcome	Randomized controlled trial or similar	>100
Stage 4 Long term	Registry, long-term evaluation	Quality assurance, identification of risk factors, comparators	Registry	>100

3. GOALS

The main goal of the research was to prove that amniotic tissue can form an appropriate scaffold for reconstructive techniques in the urinary tract. We aimed to develop a standardized and manufacturer-independent process for amnion graft preparation, ensuring sterilization and keeping the extracellular matrix intact. We chose the unseeded membranes as previous studies had demonstrated better performance of unseeded scaffolds in the urinary tract, probably due to missing graft reaction [Engel, 2014]. The preserved extracellular matrix and mesenchymal stem cells were assumed to be the key factors in regeneration. Furthermore, the seeding process is a costly and time-consuming process and complicates the manufacturing process. We also aimed to ensure that amnion is feasible for the process of cutting and suturing in surgery. Having achieved these goals, we conducted experimental studies on the immunogenicity of xenologous transplanted multilayered human amniotic membrane

in Sprague Dawley rats. In further steps, an animal model was designed to explore the functional and histological efficacy of an amnion graft for the repair of bladder and colon defects. Finally, following the IDEAL-D framework, the possibility of transfer of experimental results into clinical practice was investigated. To meet these goals, we designed the following studies.

Study I. The main purpose was to develop an *in vitro* approach for amnion preparation and to preserve the ECM integrity. Different sterilization and suture techniques were tested to achieve a perfect hold and processing of HAM [Barski, 2015; Barski, Surg Innov 2017; Barski, Int J Med Sci 2017].

Study II. We aimed to investigate the possibility of bladder and colon grafting and the immunogenicity of processed HAM xenografts in Sprague Dawley rats. A small defect was set at the bladder dome or caecum in a total of 48 animals and repaired by either suture or grafting with a multilayer HAM from a Caesarean section. Bladder volume capacity, adhesions and leakage after grafting were measured. Peri- and early postoperative complications were assessed. Histological and immunohistological analyses were performed to identify HAM degradation, graft rejection and ingrowths of surrounding tissue 7, 21 and 42 days after the implantation [Barski, Surg Innov 2017; Barski, Int J Med Sci 2017].

The goal of **Study III** was to translate the preliminary *in vivo* animal results into a clinical scenario. HAM allografts were used to treat a complex case of a vesico-vaginal fistula and a chronic wound with an entero-cutaneous fistula, and the patients were followed up in a registry for one year [Barski, 2015].

4. MATERIALS AND METHODS

4.1 Amnion preparation

HAMs were obtained immediately after elective Caesarean sections with normal gestation and informed consent from the patients. The donors were screened for infections including HIV, hepatitis and syphilis. First, the placental tissues were macroscopically evaluated to verify their suitability for subsequent tissue processing. Next, procedures were performed at room temperature and under sterile conditions. Amnion was detached from the chorion by simple mechanical traction (**Figure 2**), using sterile forceps and a scalpel. The separated amniotic membranes were washed with a sterile physiological solution, phosphate-buffered saline (PBS), and blood clots were removed. Subsequently, HAM was cut into segments of at least 5x5 cm². Larger

membranes were difficult to harvest because of varying amnion quality and fragility. After several rinsing steps, the HAMs were fixed to a sterile silicon scaffold with the epithelial side up and placed in a sterile container with 50 ml PBS. The flexible, sterile silicon scaffold (Bess GmbH, Germany) was adapted from oral surgery, where it is used for tamponades. Different freezing techniques were tested to ensure the storage of amnion. The preservation of ECM was compared in fresh amnion (fHAM) and processed amnion (pHAM) samples with storage at -20°C and -80°C. Due to the best balance of storage and ECM preservation (Study I), HAMs were frozen at -20°C until further use. (They can be stored for at least six months without signs of degradation.) For further processing, the HAMs were thawed in water and sterilized. If rinsed in PBS without sterilization, we found bacterial contamination after several days. We tried glycerine (85%) and peracetic acid (0.25%), and the best ECM preservation was found after peracetic acid (0.25%) and alcohol mixture sterilization (Wofasteril, Kesla AG, Germany). Afterwards, HAMs were incubated for 2h on the shaker. After rinsing, the HAMs were prepared in several layers applied on a sterile silicon scaffold and dried under laminar flow (**Figure 2**). Different numbers of layers were attempted to achieve the best processing of the amnion with the option of cutting and suturing. For the best hold, 4-0, 5-0 and 6-0 Monocryl (Ethicon, Norderstedt, Germany) single sutures were used at three to four points to fix HAM to the scaffold [Barski, 2015] (**Figure 2**). These monofilament and resorbable sutures are optimal for the reconstruction of the urinary tract, as they resorb slowly (about 100 days vs. 60 days for Vicryl multifilament suture) and present less scarring compared to multifilament sutures. Monocryl suture stretches more than Vicryl and PDS at higher loads and this is important due to high pressures in the bladder [Weld, 2008].

Figure 2. A. Preparation of amniotic membrane with dissection from the chorion under laminar flow. B. Washing and fixing of amniotic membrane to scaffold [Barski, 2015].

4.2 *In vitro* amnion studies

Fresh human amniotic membrane (fHAM) and processed frozen, sterilized and dried HAM (pHAM) samples from ten patients after Caesarean section were fixed in 10% formalin, embedded in paraffin, sectioned into 5 µm thick cross-sections and stained with H&E according to standard protocols. The integrity of ECM was analysed by the presence of specific cell surface antigen expression of CD90, CD105 and collagen types IV, V and VII. Immunostaining was performed with polyclonal rabbit antibodies (Abcam, USA) and antirabbit secondary antibodies (Vector Laboratories, USA). To determine whether cells within fresh amnion and processed amnion tissue proliferate, the tissues were stained for Ki-67 (Abcam, USA). Haematoxylin was used to counterstain the nuclei.

4.3 *In vivo* experiments

4.3.1 *Animals*

The animal experiments were conducted at the Institute for Surgical Research of the University of Szeged, Hungary, in accordance with the National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals). The experimental protocol was approved by the Animal Welfare Committee at the University of Szeged (license number V./146/2013) and the National Scientific Ethical Committee on Animal Experimentation (Hungary's national competent authority), and were carried out according to EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes. Forty-eight three-month-old 320–380 g male Sprague Dawley rats were used. The animals had free access to food and water and were cared for by a trained keeper and routinely inspected by a veterinarian at the University of Szeged's experimental animal facility [Barski, Surg Innov 2017; Barski, Int J Med Sci 2017].

4.3.2 *Amnion graft implantation in the bladder and colon*

The sterile procedures were performed by two surgeons using microscopic and microsurgical techniques [Barski, Surg Innov 2017; Barski, Int J Med Sci 2017]. The rats were anaesthetized with 40 mg/kg 10% ketamine, and the abdomen was shaved and prepared with an ethanol–propanol solution. A midline laparotomy was performed, the bladder was identified, and its capacity (in ml) was determined in a standardized way (Figure 3). Briefly, bladder pressure was evaluated with a cystometric method adapted from Lundbeck et al. [Lundbeck, 1989]. The dome of the bladder was exposed, a 20 G needle was inserted through a small puncture, and the

bladder was emptied with a syringe. The needle was attached to an infusion system at a 20 cm height above the bladder, and room-temperature saline was infused by gravity. The bladder capacity was measured at the time point of the infusion stop. The bladder was emptied, the procedure was repeated two additional times, and the mean value was recorded. Subsequently, a defined 0.5 cm lesion was cut at the bladder dome. In the colon group, the caecum was identified, and a defined 0.5 cm length of the caecum wall was resected. In the treated amnion group (bladder Ba, n=18, and colon Ca, n=18), a multilayer amnion patch was trimmed to cover the defect size (10x10 mm) and fixed to the bladder or colon wall with 6-0 Monocryl (Ethicon, Norderstedt, Germany) single sutures at three to four points. Additional human fibrin glue (Evicel, OMRIX Biopharmaceuticals Ltd., Israel) was used to seal the lesion. In the first control group (B1, n=6, and C1, n=6), the defect was closed with a single Monocryl 6-0 running suture and fibrin glue. In the second control group (B2, n=3, and C2, n=3), the amnion graft was sutured to the bladder or colon wall with no prior lesion. Fluid loss was compensated for by administering 3 ml of 0.9% saline intraperitoneally at the end of surgery. Subsequently, the abdominal fascia and skin were closed in layers with absorbable Vicryl 5-0 running suture and Monocryl 4-0 interrupted suture (Ethicon, Norderstedt, Germany) [Barski, *Surg Innov* 2017; Barski, *Int J Med Sci* 2017].

Figure 3. Preparation and identification of the urinary bladder in the rat.

4.3.3 Explantation and macroscopic studies

The animals were sacrificed at one week (Ba and Ca, n=10; B1 and C1, n=4; B2 and C2, n=2), three weeks (Ba and Ca, n=12; B1 and C1, n=4; B2 and C2, n=2) and six weeks (Ba and Ca, n=10; B1 and C1, n=4; B2 and C2, n=2) after surgery. Bladder capacity was determined again; subsequently, tissue samples (urinary bladder or colon, kidneys and spleen) were harvested and stored in 10% formalin solution for 2h and then stored in PBS at 4°C. HAM grafts and the bladder wall were assessed by two

surgeons for colour, tissue contraction, inflammation and pliability. The existence of peritonitis, abscess and adhesion formation was recorded. The colon wall was assessed for adhesions and inflammation. The adhesion-covered area was graded semiquantitatively between 0 and 3 according to the score developed by van der Ham et al. [van der Ham, 1992] (Table 2). The results were documented with photographs as well [Barski, Surg Innov 2017].

Table 2. Adhesion grading scale [van der Ham, 1992].

Grades	
0	No adhesions
1	Minimal adhesions, mainly between the omentum and the bowel graft
2	Moderate adhesions, i.e., between the bowel graft and the omentum or a loop of the small bowel or the abdominal wall
3	Severe and extensive adhesions, i.e., between the bowel graft and several loops of small bowel and the abdominal wall, including abscess formation

4.3.4 Histological and immunohistological studies

All specimens were fixed and embedded in paraffin. Deparaffinized sections (5 μ m) were used for staining with H&E to visualize tissue architecture and cell infiltration. Tissue sections were deparaffinized, rehydrated and subjected to a heat-induced antigen retrieval (citrate buffer, pH 6.0), followed by 3% hydrogen peroxide and avidin-biotin blocking. Prior to incubation with the primary antiserum, sections were incubated with a PBS blocking solution containing 10% normal horse serum, 0.1% bovine serum albumin, 0.05% thimerosal, 0.01% NaN₃ and 1% Triton X-100. Primary and secondary antisera were diluted in a blocking solution without Triton X-100. Sections were incubated overnight with the primary antibody and immunostained with the streptavidin–biotin peroxidase method, followed by a diaminobenzidine (DAB) chromogen solution. Finally, sections were counterstained with haematoxylin and mounted in a xylene-based mountant. Negative controls were incubated in a blocking solution without primary antibodies. Digital images of H&E and α -actin were used to evaluate smooth muscle content within the reconstructed wall. Pictures were taken using a Zeiss Axiophot microscope (Zeiss, Jena, Germany) equipped with an Olympus DP70 digital camera (Olympus, Münster, Germany) at 4x magnification. Particular

attention was paid to the slides showing the transition zone between the amnion graft and normal bladder or colon wall. A semiquantitative score from 0 to 3 for inflammation and from 0 to 2 for vascularization was used. Inflammation of the implant region was scored by counting lymphocytes in ten fields of 0.25 mm^2 in three observer-randomized H&E slides (semi-quantitative score: 0 = < 5% cells/field; 1 = 5–25%; 2 = 25–50%; 3 = >50%; 200x magnification). A similar score was used for vascularization (0 = 0 vessels/ mm^2 , 1 = 1–3 v/ mm^2 ; 2 = >3v/ mm^2 ; 200x magnification). HAM thickness was measured in μm to assess degradation and inflammation. Kidney and spleen specimens were analysed for signs of transplant rejection.

4.3.5 Statistics

The data were recorded in Microsoft Excel software and then transferred into a GraphPadPrism6.0 (Graphpad Software, Inc.) database for statistical analysis. Continuous data were checked for normality of distribution before choosing between parametric and non-parametric tests. The results were presented as medians with range or means with standard deviation (SD) in the case of a normal distribution. Data from different groups were compared using the Mann–Whitney U test and the Kruskal–Wallis test. Statistical significance was assumed at $p < 0.05$.

4.4 Clinical studies

4.4.1 A vesico-vaginal fistula

A 64-year-old female was admitted to the Department of Urology, Lukas Hospital Neuss, Germany, with sigma diverticulitis and a chronic vesico-vaginal fistula (VVF). She had a previous history of cervical cancer and radio-chemotherapy for anal cancer with complete remission. The resection of the sigma bowel and fistula were performed in our Department of Surgery. However, VVF persisted after multiple abdominal operations. The patient suffered from permanent incontinence and local skin infection. After a recovery period of three months, we re-evaluated the patient with cystography, cystoscopy, ureteropyelography and a vaginal examination. A complex vesico-vaginal fistula of 1.5 cm was detected at the apical anterior vaginal wall leading to the bladder base (**Figure 4**). Additionally, scarring of the vagina and distal ureters was found, and the patient suffered from persistent MRSA colonization of the urine. Due to the complex situation, we decided to start an individualized treatment strategy with the off-label use of HAM. Amnion preparation steps have been described previously (see Chapter 4.1). Prior to surgery, three layers of dried AM were fixed together with a 4-

0 Monocryl suture. During surgery, a single-shot antibiotic was administered and an extraperitoneal abdominal approach was chosen to allow maximum exposure. The fistulous tract and all devitalized tissue were excised sparingly. A multilayer HAM of 4 cm was used as a graft to close the defect. It was fixed with 4-0 Monocryl sutures at the edges of the normal bladder wall. We would have preferred to use a vascularized patch of either omentum or peritoneum in addition to the amniotic membrane, but this was not possible in this case. The anterior bladder wall was then closed and ureteral stents were led out in a suprapubic region to dry the bladder. Topical oestrogen was prescribed for three weeks after surgery. Anti-muscarinic therapy was started to restore bladder capacity [Barski, 2015].

Figure 4. Preoperative examination A. Cystography showing the vaginal contrast leakage and indwelling ureteral stents. Cystoscopy showing the fistula canal at the bottom of the bladder.

4.4.2 An entero-cutaneous fistula

An 83-year-old female underwent a radical cystectomy with a cutaneous ureterostomy for locally invasive bladder cancer at the Department of Urology, Lukas Hospital Neuss, Germany. Two weeks after the surgery, she was admitted again with a severe infection of the laparotomy wound. A wide resection of subcutaneous tissue with vacuum sealing was necessary, followed by a displacement plastic skin repair. A week later, the patient presented with a cutaneous fistula with purulent secretion of 30–50 ml/day. A CT scan showed the communication with a small bowel loop. The patient underwent parenteral nutrition and octreotide injections for one week with the reduction of fistula secretion to 20–30 ml/day. Due to the complex situation, we

designed an individualized treatment strategy with the off-label use of HAM dressing. The wound was measured and cleaned. A two-layer amnion dressing was applied weekly after cleaning and debridement for four weeks. A non-adherent inner dressing with Cuticell wound gauze (BSN medical, Hamburg, Germany) was then applied over the graft as well as a standard dry dressing as the outer layer. The patient was advised not to remove the dressing and to keep the dressing dry until the next visit. The wound size and secretion were documented with photographs every week. A small biopsy at the wound margin was performed at week 4. The patient provided written consent for the treatment, the biopsy and the use of photographs.

5. RESULTS

5.1 *In vitro* experiments

To determine the effect of amnion processing on tissue structure, processed pHAMs with freezing at -20°C and -80°C were compared with fresh tissues using histological H&E staining. pHAM at -20°C maintained tissue thickness and structural integrity, comparable with the amniotic layer of fresh tissue. Staining for mesenchymal cell surface antigens CD90 and CD105 and ECM collagens IV, V and VII were positive for pHAM at -20°C and fresh amnion. However, pHAM displayed no positive staining for Ki-67 (**Table 3**).

Table 3. ECM analysis of fresh amnion (fHAM) and processed amnion (pHAM) at -20°C and -80°C.

	fHAM	pHAM -20°C	pHAM -80°C
Amnion thickness (µm)	82 (60–125)	76 (55–95)	55 (33–75)
CD90	++	+	-
CD105	++	+	-
Collagen IV	++	++	+
Collagen V	++	+	-
Collagen VII	++	+	+
KI67	+	-	-

Amnion thickness is presented as median and range. Immunostaining was semiquantitatively assessed as ++, strong staining >50%, +, staining 10–50%, -, staining <10%.

5.2 *In vivo* amnion xenograft bladder reconstruction

5.2.1 Clinical course and functional results

Two animals (11%) died in the treated group, one due to postoperative sepsis, another during anaesthesia. No animals from the control groups died. No other complications higher than grade II (Clavien–Dindo classification) were observed [Clavien, 2009]. The bladder capacity did not change in the treated group but decreased significantly in control group B1 with a suture of the lesion ($p=0.01$) (**Figure 5**) [Barski, *Int J Med Sci* 2017].

Figure 5. Bladder capacity pre- and postoperatively in amnion-treated group Ba and control group B1. The bladder capacity decreased significantly in the control group due to scarring of the sutured lesion (* $p<0.05$).

5.2.2 Macroscopic examination

No signs of severe inflammation were found in the abdominal cavity during reoperation. Meso-adhesions to the HAM graft were detected in most treated cases. HAM appeared as a thick, oedematous graft with inflammation running from the middle towards the transition zone of the bladder wall at day 7. At days 21 and 42, HAM was still well defined, albeit with reduced inflammation (**Figure 6**). Adhesions were present in some cases. Inflammation was less prominent in the control groups.

Figure 6. Macroscopic and histological evaluation (H&E staining) of inflammation and AM degradation over time (days 21 and 42 after grafting). Decreased inflammatory cell infiltration (*) and increased vascularization (V) in the periamniotic transition zone. No signs of AM degradation. Scale bar 200 μ m.

5.2.3 Microscopic examination

The xenotransplanted HAM graft covered the bladder wall and maintained its architecture at day 7. The lesions could be recognized as regions without smooth muscle cells but with abundant connective tissue and signs of inflammation. Significant inflammation and increased numbers of blood vessels were observed in the amnion and between the amnion and the adventitia of the bladder, which resulted in an enlarged amnion. Infiltrated lymphocytes agglomerated mostly in the area bordering the HAM (**Figure 6**). At day 21, the amnion appeared less thick and the inflammation was significantly diminished ($p < 0.05$) (**Figures 7, 8**). New capillaries started to grow into the surrounding connective tissues, and scattered smooth muscle cells emerged in the area of the lesion (**Figure 6**). Signs of inflammation mostly disappeared in the control groups. At day 42, it became more difficult to discern the region of the lesion, where the amnion formed a thinner layer on the bladder wall with no signs of degradation (**Figure 6**). Inflammation was markedly reduced in the amnion and in the zone between the amnion and the bladder wall ($p < 0.05$) (**Figure 6**). The number of large vessels in the amnion appeared to be reduced and periamniotic vascularization increased, but these results were not significant. Connective tissue, bundles and thin muscle layers were found in abundance in all the groups. Smooth muscle regeneration occurred more rapidly in the amnion group, although the

difference in the regeneration was not significant ($p < 0.05$) compared to the control group with a suture of the lesion.

Figure 7. Reduction of inflammation over time. Semiquantitative analyses of inflammatory cells. Ba: Amnion repair of bladder defect; Control/Lesion (B1): Closure of bladder defect with suture; Control/Amnion (B2): Amnion bladder onlay without defect; Semi-quantitative score: 0 = <5% cells/field; 1 = 5–25%; 2 = 25–50%; 3 = >50% with 200x magnification. Data are expressed as median with range. * $p < 0.05$.

Figure 8. Changes in amnion thickness over time (days 7, 21 and 42). Ba: Amnion repair of bladder defect; Control/Amnion (B2): Amnion bladder onlay without defect. Data are expressed as mean with SD, $p < 0.05$.

5.3 *In vivo* amnion xenograft colon reconstruction

5.3.1 Clinical course and functional results

Two animals (11%) died in the treated group, one animal due to postoperative sepsis and the other during the anaesthesia, and were hence excluded from the analysis. No animals from the control groups died. These were the same animal losses as in the bladder group. No severe complications higher than grade II (Clavien–Dindo classification) were observed [Barski, *Int J Med Sci* 2017].

5.3.2 Macroscopic examination

No signs of severe inflammation were found in the abdominal cavity during reoperation. The HAM appeared as a thick oedematous graft infiltrated by signs of inflammation. The inflammation was less severe in the control groups. Strong adhesions of the HAM graft to the small bowel and abdominal wall that withstood tractions were detected in most treated cases (**Figure 9**). At day 7, a higher adhesion score with a larger coverage area was found in the amnion group (1.8 ± 0.45) vs. group C1 (0.5 ± 0.7) ($p < 0.05$) (**Figure 10**). However, similar adhesion scores were detected for C1 and C2. At day 21, the adhesion score was higher in the amnion group (1.8 ± 0.84) vs. group C1 (1 ± 0) ($p = 0.178$) but did not reach the level of significance. HAM was difficult to recognize and in some cases was not detectable at day 42. The adhesions increased in the amnion group vs. groups C1 and C2 ($p = 0.052$) (**Figures 10, 11**).

Figure 9. Macroscopic evaluation of adhesion in amnion-treated group A (see Table 2). **A.** Strong adhesions to small bowel and abdominal wall with oedema and inflammation of the lesion site were detected in the amnion-grafted group (7 days). **B.** Moderate adhesions without signs of inflammation were detected in the control group with a suture of the lesion (42 days).

Figure 10. Adhesion formation in treated amnion group A, control group C2 with amnion onlay with no lesion and control group C1 with a suture of the lesion. Time point 1: 7 days; time point 2: 21 days and time point 3: 42 days. Plotted are means $\pm$ standard errors. Two-way ANOVA test ($p < 0.05$) for time points 7, 21 and 42 days. A higher adhesion score was found in the amnion group vs. group C1 ($p < 0.05$ for time point 1) and a slight increase of adhesion in the amnion group over time.

5.3.3 Microscopic examination

At day 7, strong inflammation with abundantly increased numbers of lymphocytes and blood vessels was observed in the amnion layers and between the amnion and the submucosa of the colon, which resulted in an enlarged amnion. A lower-level, but still strong inflammation was found in control groups C1 and C2 (**Figures 11, 12**). HAM thickness was reduced and inflammation was significantly diminished ($p < 0.05$) at days 21 and 42 (**Figure 12**). Connective tissue bundles and scattered smooth muscle cells appeared in the area of the lesion (**Figure 11**). In control group C1, signs of inflammation (the presence of lymphocytes) had mostly disappeared and there were also no clear signs of regeneration of smooth cells in the lesion region. At day 42, it became more difficult to verify the presence of the amnion in the treated group; the different layers of the amnion could no longer be distinguished. The amnion, which was significantly reduced in thickness, formed a thinner layer on the colon wall (**Figures 11, 13**). In the control group with no lesion (C2), the amnion was completely degraded and could no longer be detected (**Figure 13**). Inflammation was markedly reduced in the amnion and in the zone between the amnion and the colon wall but was still significantly higher compared to control group C1 ($p < 0.05$). Despite the presence

of scattered smooth muscle cells and bundles, it was difficult to measure if there was a clear regeneration of smooth muscle compared to control group C1 (**Figure 11**).

Figure 11. Histological evaluation of AM degradation and inflammation in the treated group over time (A: 7 days and B: 42 days after grafting). AM, amniotic membrane; Muc, colon mucosa. Arrows show AM thickness. Significant AM degradation and reduction of inflammation over time. Decreased inflammatory cells and increased vascularization in periamniotic transition zone.

Figure 12. Reduction of inflammation over time, semiquantitative analyses of inflammatory cells. Plotted are means $\pm$ standard errors. T1: 7 days; T2: 21 days; T3: 42 days; Group Ca; amnion repair of colon lesion; Control/Amnion: Group C2; amnion only without lesion; Control/Lesion: Group C1; closure of colon lesion with suture. Semiquantitative score: 0 = <5% cells/field; 1 = 5–25%; 2 = 25–50%; 3 = >50%; 200x magnification. A significant decrease of inflammation in the treated group T2 vs. T1 was detected ($p < 0.05$). However, the presence of inflammatory cells was significantly higher in the treated vs. control/lesion group at every time point ($p < 0.05$).

Figure 13. Thickness of the multilayer amniotic membrane (μm). Plotted are means $\pm$ standard errors. T1: 7 days; T2: 21 days; T3: 42 days. Group Ca; amnion repair of colon lesion; Control/Amnion: Group C2; amnion onlay without lesion; A significant reduction of HAM thickness over time T3 vs. T1 in both groups ($p < 0.05$).

5.4 Graft rejection

No macroscopic signs of rejection were found in the kidney and spleen specimens. However, four out of six animals in the treated group showed an affected kidney at day 21. To rule out an obstruction or increased bladder pressure due to the HAM graft as the cause of these findings, we compared the results with the study, where we used an amnion graft on the colon. The same results were observed. The changes were subtle with slightly enlarged tubuli and a slightly enlarged urinary space. The glomeruli appeared denser. No immune cells or other signs of transplant rejection were found. At day 42, only the kidneys were still slightly affected in two out of five animals, whereas no such changes were detected in the controls without amnion. All things considered, the presence of a transient, subtle transplant glomerulitis is suggested in the rat HAM grafts.

5.5 Clinical cases

5.5.1 A vesico-vaginal fistula

Seven days after surgery, a cystography was performed showing no leakage. In the following three months, a re-evaluation with cystography, cystoscopy and a vaginal examination was performed with no signs of recurrent fistulas, and full recovery of the vaginal epithelium was present (**Figure 14**). The ureter stents were internalized, and the patient was able to micturate without incontinence. The bladder capacity was restored to a volume of 250 ml. No severe complications and no signs of graft rejection were observed during the six-month follow-up. The MRSA colonization in the urine

was successfully eradicated. However, during the ten-month follow-up, the patient reported vaginal urine leakage again and a recurrent fistula was observed at the margin of the amnion graft.

Figure 14. Postoperative examination three months after surgery. **A.** Cystography showing no contrast leakage, indwelling ureteral stents and catheter. **B.** Cystoscopy showing the amnion graft. HAM merged with the surrounding bladder tissue at the margins but was still clearly demarcated with no signs of overgrowing urothelium or stroma. *Fixation points with suture.

5.5.2 An entero-cutaneous fistula

The wound size at first HAM application was 3x2x1 cm (**Figure 15**). Dressings and off-loading were conducted as described above. After two weeks, the secretion stopped and the ulcer decreased in size by 20% (**Figure 15**). The ulcer healed after eight weeks with four applications of HAM. The wound remained healed at six-month and nine-month follow-ups. The histological analysis showed re-epithelialization and muscle cell recovery four weeks after the treatment. HAM was not degraded and was still detectable as a smooth layer on the surface. Moderate inflammation was present, but graft rejection was not observed (**Figure 16**).

Figure 15. A. Initial presentation with entero-cutaneous fistula and wound ulceration. B. After two weeks, the secretion stopped and the wound shrank by 20% and contracted. HAM dressing is marked with arrow.

Figure 16. Histological analysis of wound healing. High magnification (400x) Vimentin-stained sections. A. Epidermis formation and dermis composition with muscle cell formation (arrows). B. Muscle cells (arrows) adhering to the scattered pieces of HAM.

6. DISCUSSION

6.1 Amnion preparation and *in vitro* studies

There are several methods for amnion preparation. However, none of them has been compared properly or standardized. There are several key points that should be discussed in this respect. For clinical use, the amniotic membrane should be intact and free from contamination. Bacteriological investigations from previous studies have shown that HAM is already sterile when collected, especially from a Caesarean

section, as in the study presented [Addis, 2001]. Nevertheless, a low risk of infection is assumed for the recipient, so different sterilization methods have been proposed in the past. Today, cryopreserved, dried, irradiated or freeze-dried amniotic tissues have been used in studies. Cryopreservation makes storage possible for six months, while the use of glycerin solution prolongs it [Maral, 1999; Tseng, 1997]. Johnson et al. compared the functionality of single-layer viable cryopreserved human amniotic membrane (vCHAM) with multilayer devitalized dehydrated human amnion/chorion membrane (dHACM) *in vitro* and *in vivo* to determine the effect of different processing methods on HAM [Johnson, 2017]. The structure and thickness of fresh amnion were retained in vCHAM but were compromised in dHACM. Only viable cryopreserved human amniotic membrane increased growth factors over time and responded to chronic wound stimuli *in vitro* and *in vivo*. In our study, we stored amniotic membranes at -20°C with PBS to deliver a safe, effective, and minimally manipulated amniotic tissue. Freezing at -80°C presented a decrease of ECM integrity and staining of collagens. The amnion was already contaminated with bacteria after harvesting. We used a commonly accepted Wofasteril (0.25% peracetic acid and ethanol mixture) method for sterilization. As compared to other methods (glycerine, silver nitrate or radiation), the Wofasteril procedure proved to be a reliable method to sterilize different biological transplants preserved by the extracellular matrix and is accepted by the Paul Ehrlich Institute (PEI), a German research institution and medical regulatory body [Pruss, 2001]. Moreover, peracetic acid did not significantly influence the cytotoxicity of biological implants and is therefore recommended for clinical use [Pithon, 2013]. Additionally, the absence of viable cells in HAM due to sterilization should prevent an immunological rejection. Our histological analyses supported the notion that the extracellular matrix and its structure were preserved by our processing method (Table 3). Fresh amnion demonstrated the best ECM preservation but at the cost of contamination, and freezing at -80°C destroyed the ECM structure. Freezing amnion at -20°C and sterilization with Wofasteril provided the best results in our studies. We confirmed the previous study reports on collagen structure and the mesenchymal cell presence in amnion [Resch, 2006]. Weak Ki67 staining confirmed the presence of avital epithelial cells. One problem that occurred was the fragility of amniotic membrane. It must be handled with care to avoid tearing and rolling. For this reason, HAM was dried under laminar flow and fixed by a suture to a sterile silicon scaffold. Moreover, we used several layers of HAM to give it better handling and more stability

for the planned tissue reconstruction [**Barski, Surg Innov 2017; Barski, Int J Med Sci 2017**].

6.2 Urinary tract reconstruction with amniotic tissue

The technique of applying HAMs in reconstructive urology was introduced for the first time in 1955 in reconstructing a urethra [**Lenko, 1955**]. However, only a few publications were available until the last decade, as HAM became more readily available due to improved preservation. A Polish group described a technique to supplement long ureteral wall strictures (5.5 cm) by using folded HAM allografts and presented good, sustainable results after an average follow-up period of 25.2 months [**Koziak, 2007**]. Brandt and his co-workers successfully reconstructed a female urethra using autologous grafts prepared from HAM [**Brandt, 2000**]. Excellent integration of the implanted amnion graft within the host urinary tract wall and reduced fibrosis were reported after the reconstructive procedures. Adamowicz and his colleagues designed a sandwich-structured biocomposite material from a frozen cell-seeded (i.e. bone marrow-derived mesenchymal stem cells) AM and covered it on both sides with two-layered membranes prepared from electrospun poly-(L-lactide-co- ϵ -caprolactone) (PLCL). The authors considered this reinforcement of the AM necessary because of its poor mechanical qualities. The new biomaterial (10x10 mm) was used for bladder augmentation after a hemicystectomy in ten rats, which were sacrificed after three months [**Adamowicz, 2016**]. Immunohistochemical analysis demonstrated effective regeneration of the urothelial and smooth muscle cells and complete PLCL degradation. However, the authors reported a moderate inflammatory reaction after three months. In contrast, the results of our study confirm previous reports in which the elasticity and durability of multilayer HAMs were described and no signs of leakage and unchanged bladder capacity were observed after the reconstruction. The inflammatory reaction had almost disappeared already after six weeks. Large grafts need sufficient nutrition of the cells and removal of waste products to eliminate/reduce the risk of fibrosis and shrinkage. Having a diffusion distance from the supplying blood vessel of ~150–200 μm , HAMs efficiently conduct sufficient oxygenation of cells by diffusion [**Laschke, 2006**]. HAM acts as a basal membrane and thus facilitates the migration and enhanced adhesion of the epithelial cells [**Sonnenberg, 1991**]. Although we expected a more rapid regeneration of smooth muscle in the amnion group due to the presence of growth factors, our results were not significant in this respect and it was not possible to compare the regeneration in a standardized way due to the small

size of the grafts used and an overlap with the normal bladder wall. No signs of graft shrinkage or necrosis were found during the six-week follow-up period. We used unseeded amniotic membranes in a three- to four-layered technique. Incorporation of autologous urothelial and smooth muscle cells involves a time-consuming and costly process of harvesting, culturing and seeding the cells. Furthermore, cells from diseased bladders may behave differently from normal cells, rendering their use for tissue engineering applications questionable [Roelofs, 2016]. Another reason for choosing unseeded membranes was small defects in the bladder. With regard to the necessity of a urothelial layer, earlier studies determined that short gaps are closed by ingrowing urothelium. Dorin et al. showed that the maximum distance for tissue regeneration is 0.5 cm [Dorin, 2008]. Moreover, previous studies found that scaffolds without seeded urothelial cells performed better than those with seeded cells [Engel, 2014].

The limitations of our study were the small size of the grafts (10x10 mm) and the short follow-up period. Regeneration in large constructs and damaged tissue conditions needs to be further evaluated. We used HAM grafts in a healthy rat population; future studies should analyse HAM performance for the reconstruction of radiated or otherwise damaged tissues.

6.3 Bowel tract reconstruction with amniotic tissue

The formation of adhesions is one of the key problems following surgical interventions in the peritoneal cavity [Brochhausen, 2011]. Several strategies for the prevention of adhesions have been proposed, including inhibition of inflammation, prevention of fibrin formation, promotion of fibrinolysis, anti-angiogenesis and tissue engineering. Allogenic (peritoneum), xenologous (collagen) and synthetic materials (polymers) and sealants (fibrin) have previously been tested to prevent adherence formation [Cueto, 2014; Hoare, 2014; Kawanishi, 2013; Wenger, 2015]. Most of the trials were animal studies and have not yet been able to identify the optimal barrier to prevent adhesions in a sustainable way and to be translated into clinical practice [Brochhausen, 2011]. We aimed to prove the application of human amniotic membrane in bowel surgery to protect anastomosis and to prevent adhesions. For this purpose, we chose a rodent model of simulated anastomosis leakage and repair of small defects in the caecum. The present study demonstrated that the use of amniotic membranes for the repair of bowel lesions in a xenograft model is not beneficial as compared to standard therapy. Although we did not find any clinical signs of rejection in our xenologous experiment, we did observe extended adhesions and an inflammatory reaction in the amnion group.

Moreover, the incidence of adhesion formation (89% in the treated group vs. 33% in the control group, $p < 0.05$) and inflammatory reactions were significantly higher in the amnion group. Similarly, an increased number of inflammatory cells was reported by other groups after the application of HAM in rodent models [Schmidt, 2010; Uludag, 2009]. We assumed that the anti-inflammatory properties of HAM would reduce the formation of adhesions. However, our results showed that the adhesions could not be prevented and were even increased in the amnion group, probably due to increased inflammation by insufficient bowel sealing with the amnion graft. Only a few groups have applied HAMs in reconstructive bowel surgery to date. Schmidt used a patch of monolayer HAM to repair an 8 mm duodenal lesion in 42 Wistar rats [Schmidt, 2010]. The animals were followed up for 28 days and adhesions were detected in all the animals. Two of the animals presented with obstruction and one animal died from peritonitis. The HAMs degraded after 14 days and the regeneration of mucosa and smooth muscle increased with time. Epithelialization of the HAMs started three days after surgery and was completed between three and four weeks after the operation. The authors concluded that HAMs can be used as a temporary seal to re-establish the duodenal wall structure. The colon bacteria digest the amnion tissue and cause the patch to degrade. Several studies have shown the degradation of the extracellular matrix components from bacterial-derived metalloproteases in the colon [Pasternak, 2010; Shogan, 2015]. If applied as a graft, a rapid degradation of the amnion is reported between 14 and 90 days, depending on the grafted tissue and number of layers [Kesting, 2008; Schmidt, 2010]. Transformation of multilayer HAMs, tissue reorganization and degradation were observed between 21 and 42 days in our experiment. In our study, fibrin glue was also used to seal the lesions in all the groups. No adverse effects of fibrin glue on adhesion formation have been reported previously [Kanellos, 2006]. Another problem with our animal model was the healthy animal population. As proposed by several groups, a peritonitis model could be appropriate to clarify the protective role of HAM on the established anastomosis [Wichtermann, 1980]. A Turkish research group induced bacterial peritonitis in 50 rats by performing a caecal ligation and puncture. Ten rats served as controls for the bursting pressure measurement, while the other 40 animals were divided into two groups (the anastomosis group or the amniotic membrane group) with all of them undergoing colonic anastomosis. The caecum anastomosis was covered with a 10 mm amnion wrap. The anastomotic leakage rate was as high as 25% in the standard anastomosis

group vs. 0% in the amnion group after a seven-day follow-up period [Uludag, 2009, 2010]. Unlike in our model, the authors reported a high dehiscence rate of 40–50% in the control group with standard anastomosis; this was probably due to the different experimental design. In our study, no signs of dehiscence were detected in the control group with the suture of a small bowel wall defect. However, the increased initial inflammation and adhesions in the amnion group appear to be a sign of insufficient bowel sealing with the amnion graft. Additionally, one case of postoperative peritonitis and sepsis was detected in the HAM group.

In conclusion, we suggest that HAMs alone are not suitable barriers to preventing adhesions and inflammation and therefore are not beneficial to a standard suture for the reconstruction of the bowel wall in a xenograft model. However, anastomosis protection with HAM application in a high-risk situation (e.g. peritonitis model) requires further evaluation.

The limitations of our study were the rather small grafts (i.e. 10x10 mm), the short follow-up, the small sample size and the xenograft itself (see below). Nevertheless, it should be added that a small-scale investigation with a small number of cases was purposefully planned as an innovative proof-of-principle study.

6.4 Limitations of the xenograft animal model

A number of research groups employ rodents as they are the standard cost-effective model for most clinical animal trials. Following the IDEAL-D recommendations, we first conducted animal experiments to clarify the possible graft rejection in a xenograft rodent model and the applicability of HAM as a future biomaterial. No clinical signs of rejection were detected in our xenologous experiment, although a transient inflammatory reaction was seen in the amniotic and periamniotic tissue. A similar transient increase in inflammatory cells was reported by Kesting et al., who used cryopreserved HAMs for soft tissue repair in rats [Kesting, 2008]. Additionally, most animals in our study's amnion group presented a subtle acellular transplant glomerulitis. These changes can be classified as borderline mild acute rejection according to the Banff classification [Bhowmik, 2010]. We suggest that the glomerulitis observed during our experiments was due to the xenologous model and would not develop in an allograft. With the use of HAM, both patients demonstrated no signs of inflammation and graft rejection. The grafts were well tolerated without side-effects. Our findings indicate that HAM is an immune-privileged tissue, especially if no viable epithelial cells are present. Positive results were reported

considering the tissue regeneration after bladder reconstructions with HAM [Barski, *Int J Med Sci* 2017]. However, we should be aware that animal results cannot be completely translated in humans. Another problem is that we worked with healthy young bladders in the animal experiments. Tissue alteration together with structural changes and impaired wound healing in the aging bladder could not be predicted. For future studies, it would be essential to simulate animal models with structural tissue changes using the animal model adapted to the patient target group. Possible future studies should aim to test different HAM graft techniques in peritonitis and fistula animal models to simulate the outcome in humans [Lindberg, 2015; Uludag, 2010].

6.5 HAM-assisted repair of a vesico-vaginal fistula

Complex vesico-vaginal fistulas usually present a poor outcome with high recurrence rates and a devastating course [Ghoniem, 2014; Hampel, 2015]. Radiation-induced recurrent vesico-vaginal fistulas have the lowest success rate in terms of successful treatment and require the most demanding interventions [Ghoniem, 2014]. The surgical approach to the repair of VVF depends on the surgeon's experience, the type and location of the fistula, and the patient's specific preferences [Ghoniem, 2014; Hampel, 2015]. The type of vascularized interposition flap is determined by how proximally (peritoneal, O'Connor approach, muscle flap) or distally (Martius labial fat) the genitourinary fistula is located in the vagina (85–100% success) [Ghoniem, 2014]. The flaps used run the risk of infection, necrosis, loss of function with time and long-term morbidity. To avoid these problems, different xeno-, homo- and autologous grafts have recently been proposed for the repair of VVF [Ghoniem, 2014; Robles, 2009]. Vascularization of the graft or flap is the main requirement for successful healing; otherwise, fistulas frequently recur. The postoperative outcome after fistula repair significantly depends on the degree of angiogenesis and inflammation. Moreover, an economically reasonable and alternative biomaterial for the treatment of VVF in developing countries with a high VVF incidence is needed. According to available reports and studies, HAM incorporates all these key determinants of a suitable biomaterial [Koizumi, 2000; Koob, 2014; Laschke, 2006; Resch, 2006]. In our case, we had a recurrent fistula at the margin of the previous defect ten months after the initial surgery. Possible reasons could be the technique used, as we employed the transvesical procedure but did not separate the vaginal wall from the bladder base and did not close the defects additionally due to the fragile tissues and close position of the fistula to the ureteral orifices. HAM was used as a sealing onlay graft, and only served

as a transient seal in damaged radiated tissue. There is only one other published report on VVF repair with an amniotic membrane allograft in an irradiated field with a robotic procedure [Price, 2016]. The vaginal and bladder fistula defects were closed separately in two layers using interrupted 4-0 polydioxanone (PDS, Ethicon). A rehydrated amniotic membrane patch (Amniofix, MiMedx, Inc., Marietta, GA, USA) was used as an interposition graft between the vaginal wall and bladder and was sutured in place over the surface of the vaginal side. However, the follow-up was shorter, unlike in our case. After five months, no incontinence or recurrence of the fistula was found. Additionally, a different processing of the amnion allograft was applied in this study [Koob, 2014]. Independent of the amnion processing, it was possible to use the membrane as a patch, and it appears to have functioned as an additional barrier that was limited in time. Another potential problem with the dehydrated allograft is the fact that the duration of the angiogenic effect *in vivo* remains unknown and patients with radiation fistulas remain at risk for recurrent fistulas for years [Price, 2016]. Further well documented and published clinical trials that share experience with the technique and HAM handling could shed light on the potential value of HAM for the treatment of VVF. Due to a rare condition, it is important that every case report be registered and reported (IDEAL Stage 1).

6.6 HAM dressing for the treatment of chronic wounds

Dehydrated human amnion/chorion membrane (dHACM) has been previously shown in randomized trials to be effective in treating chronic diabetic foot ulcers (DFU) [Laurent, 2017]. In a prospective, randomized, single-centre clinical trial, Zelen et al. compared wound reduction and rates of complete healing in patients with diabetic foot ulcers treated with dHACM versus standard of care. Significant differences were observed in wound reduction at four and six weeks, with wounds decreasing in size by a mean of $32 \pm 47.3\%$ in the standard of care group ($n=12$) versus $97.1 \pm 7\%$ ($P < 0.001$) for the patients receiving dHACM ($n=13$) [Zelen, 2013]. Another prospective randomized trial of 40 patients with chronic DFUs reported an average time of 2.4 weeks until complete healing in 90% of DFUs, with dHACM allografts were applied weekly [Zelen, 2014]. In the first week, we changed the external dressings every two days due to the initial infection of the wound. Amnion acts as a physical barrier against bacterial contamination and creates the moist environment required for healing [Mao, 2016]. However, most of the reported studies analysed use of HAM and HACM in clinically uninfected cases. Only one other study randomized a tissue-engineered form

of wound dressing containing acellular human amniotic collagen membrane vs. wet dressing in patients with partially infected wounds. The complete healing rate was significantly higher for the amnion group (40.7% vs. control group, 16.7%) [Mohajeri-Tehrani, 2016]. Moreover, HAM is cost-effective in the treatment of chronic wounds and reduces hospitalization [Mohajeri-Tehrani, 2016; Zelen, 2014]. In conclusion, we were the first group to report on the successful treatment of an entero-cutaneous fistula and infected wound with HAM. HAM is simple to use and allows us to cover larger and irregular surface area wounds as well as treating deep tunnelling wounds. Future studies should be conducted with larger numbers of patients with infected wounds. Inclusion of amnion dressings in the algorithm of secondary wound care should be evaluated in the future.

6.7 The project in terms of the IDEAL-D recommendations

Animal studies (IDEAL-D Stage 0) represent safe, cost-effective options to optimize reconstructive techniques with HAM (onlay, interposition graft, different layers) and for different indications. Results from animal and cadaveric studies (Stage 0) cannot be directly translated to human studies but should be considered as preliminary models for the biological performance of implant materials. Protection of intellectual property at the early stages of development should be regulated by the authorities [Sedrakyan, 2016]. A functionally useful description was presented for the repair of VVF and an entero-cutaneous fistula with HAM (Stage 1). This procedure should prevent surgeons and researchers from repeating a harmful error reported by another investigator and bundle the knowledge. Prospective, quasi-randomized clinical development studies (Stage 2) with the application of HAM for ureteral reconstruction, protection of the urethra anastomosis and repair of fistula conditions in the urinary tract are planned for the next step. These studies will be able to describe the technique modification in surgical procedures and establish a consensus among surgical teams ($n < 100$). At this stage, the technique is performed by an investigator and a few other innovators, and the aim should be approval by regulatory authorities. The main outcome criteria and complications should be identified and reported using a validated classification. If possible, randomized trials are recommended at IDEAL Stage 3. Data should be included in a registry at an early stage to uniquely identify the patient and to enable surgeons to track outcomes and adverse events and to vet findings from smaller observational studies (Stages 2–4). Our research group used an international registry and our own web-based one [Agha, 2017; Barski, *Int J Surg* 2017]. The main

problem at this stage is the challenging technical requirements for producing TE materials. Controlled manufacturing sites are required according to the principles of Good Manufacturing Practice. Such issues become even more challenging when seeded matrices are used. In Europe, the process is constrained by regulatory challenges because of legislation in individual countries within the EU that are regulated by the European Medicines Agency [**Ram-Liebig, 2015; Sievert, 2017**]. We need a safe regulatory system that encourages the production of adequate valid evidence on safety and efficacy and does not hinder innovation unnecessarily. IDEAL-D could provide a framework for the regulation of TE studies and innovation in the future.

7. SUMMARY OF THE NEW FINDINGS

The development of this surgical device (application of HAM in reconstructive urology) strictly followed the IDEAL-D recommendations at every step to ensure comparability and transparency.

1. A sterilization process was developed. Then, several layers of HAM were attached to a silicon scaffold to achieve stability and better handling during surgery. The ECM structure and collagen compositions were preserved with the processing method, which was proven histologically.
2. Unseeded HAM grafts were used to reconstruct the urinary bladder in a short-term *in vivo* xenograft study. No signs of graft shrinkage or necrosis were present during a six-week follow-up period, and only a minimal immunological reaction developed. The functional and histological results demonstrated the suitability of HAM for reconstructing the rat urinary tract.
3. Unseeded HAMs increased adhesion formation and were not suitable for bowel augmentation in the rat xenograft model. HAM grafts are not indicated for the reconstruction of the bowel wall. The anastomosis-protective property of HAM in high-risk situations (e.g. peritonitis models) requires further evaluation.
4. We demonstrated the possibilities for the use of HAM in clinical practice as a transient sealing onlay graft. However, recurrence developed ten months after the HAM-assisted repair in the case of a complex vesico-vaginal fistula. Another clinical case with an entero-cutaneous fistula and infected wound was successfully treated with several HAM dressings. HAM was simple to use and allowed us to cover large and irregular surface areas and to treat deep tunnelling wounds. This suggests that HAM can be a useful, potential scaffold for reconstruction surgeries of the urinary tract.

8. ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my scientific and clinical mentor, Professor Thomas Otto, head of the Urology Department at Lukas Hospital Neuss, for his scientific guidance and at the same time for offering such immense scientific opportunities.

I am especially grateful to Professor Mihály Boros, head of the Institute of Surgical Research at the University of Szeged, for his constant support and encouragement throughout this entire project. His optimistic and fruitful attitude has fundamentally helped me to establish and conclude this international collaboration project.

I am also particularly grateful to Gabriella Varga, PhD, senior lecturer at the Institute of Surgical Research at the University of Szeged, for her excellent technical assistance and amicable collaboration during the experiments. Many thanks as well to Isabel Pintelon, PhD, Laboratory of Cell Biology and Histology, University of Antwerp, for her assistance in providing histological and immunohistological studies and for her continuous scientific input throughout the project. Furthermore, I wish to express my deepest gratitude to Professor Jean-Pierre Timmermans and Professor Peter Ponsaerts, University of Antwerp, for their valuable collaboration and support during the project. I owe a debt of thanks to my co-worker and friend, Holger Gerullis, MD, PhD, University Hospital for Urology, School of Medicine and Health Sciences, Carl von Ossietzky University, Oldenburg, for his enormous assistance during the animal experiments as well as his valuable suggestions, which have contributed considerably to the increased scientific value of the studies in this PhD thesis. I am also most grateful indeed to Albert Ramon from ITERA for his invaluable networking support over the last decade and during this project in particular. In addition, I greatly appreciate the continuous help and clinical and scientific input I have received from a general surgeon's point of view from Professor Peter Goretzki and Bernhard Lammers, Department of Surgery, Lukas Hospital Neuss. Many thanks as well to Professor Peter McCulloch and Professor Art Sedrakyan from the IDEAL collaboration group for the valuable scientific support and the opportunity to discuss our project during the IDEAL conference. I also wish to express my gratitude to Thorsten Ecke, PhD, Department of Urology, Helios Hospital, Bad Saarow, for the successful collaboration on this project. Annette Wiggen-Kremer, Laboratory of Tissue Engineering, Neuss, deserves particular gratitude for her persistent motivation and outstanding achievement in the majority of the *in vitro* experiments.

Very special thanks are reserved for my wife Lillie. Without her ongoing support, patience, optimistic attitude and love, this PhD study could not have been completed. Last, but not least, I wish to thank my parents for their love, patience and trust.

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ANNEX

- I. Barski et al. Central Eur J Urol (2015)
- II. Barski et al. Int J Med Sci (2017)
- III. Barski et al. Surg Innov (2017)
- IV. Barski et al. Int J Surg (2017)

SHORT COMMUNICATION

TRAUMA AND RECONSTRUCTIVE UROLOGY

Repair of a vesico-vaginal fistula with amniotic membrane – Step 1 of the IDEAL recommendations of surgical innovation

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Citation: Barski D, Gerullis H, Ecke T, et al. Repair of a vesico-vaginal fistula with amniotic membrane – Step 1 of the IDEAL recommendations of surgical innovation. Cent European J Urol. 2015; 68: 459-461.

Article history

Submitted: July 27, 2015

Accepted: Oct. 21, 2015

Published on-line: Nov. 13, 2015

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Introduction Complex vesico-vaginal fistula (VVF) has a high recurrence rate and so the repair with graft tissues seems to be favorable. Amniotic membrane (AM) plays an increasing role as a scaffold for the repair of defect tissue due to its unique biological properties with regard to promoting wound healing.

Material and methods An innovative surgical procedure for AM-assisted repair of a complex vesico-vaginal fistula as the Idea Stage following the IDEAL recommendations is presented. The development of amnion preparation and the involved surgical steps are described.

Results We are able to report a successful repair of VVF by abdominal approach with an amniotic membrane graft. Good functional results, no adverse events and no graft rejection have been detected.

Conclusions Favorable results confirm the technical simplicity, safety and efficacy of this procedure. Following the IDEAL recommendations, consecutive animal experiments and a cohort study are in progress.

Key Words: amniotic membrane ↔ vesico-vaginal fistula ↔ IDEAL recommendations

INTRODUCTION

Vesico-vaginal fistulae (VVF) are frequently caused by complex onco-gynecologic or pelvic surgery and may be aggravated by oncologically necessary radiotherapy. These procedures are known to lead to symptoms such as permanent incontinence, leakage of urine from the vagina, infection and pain; therefore, significantly deteriorating the quality of living for the affected women [1]. In addition, numerous surgical procedures (in several cases requiring multiple interventions) for fistula repair like urinary diversion, etc. may worsen the situation for these patients [2]. New individual approaches with graft applications are therefore indicated to improve the local anatomical conditions and to reduce the symptoms. Several biological and artificial materials have been used,

but can lead to further complications such as rejection, necrosis and infection. First reports of amnion application into the pelvic region have shown fast epithelialization and improved functionality in vaginal and bladder reconstruction, but, so far, there is no standardized procedure [3, 4].

Patient-tailored innovative surgical approaches are difficult to standardize and respective recommendations cannot easily be generalized. A new approach, the IDEAL method has been proposed in 2009 by McCulloch and colleagues [5]. The IDEAL procedure clearly provides stages of surgical innovations, which allow for the ability to assign a new method to its specific level of development and evidence. For the first time, we present a VVF repair with amniotic membrane as the “Idea level” following the IDEAL recommendations.

MATERIAL AND METHODS

A 64-year old female was admitted to our hospital with sigma diverticulitis and chronic vesico-vaginal fistula (VVF). She had a previous history of cervical cancer and radio-chemotherapy for anal cancer with complete remission. The resection of the sigma-bowel and fistula were performed in our department of surgery. However, VVF persisted after multiple abdominal operations. The patient suffered due to permanent incontinence and local skin infection. After a recovery period of 3 months, we reevaluated the patient with cystography, cystoscopy, ureteropyelography and vaginal examination. Complex vesico-vaginal fistula of 1.5 cm was detected at the apical anterior vaginal wall leading to the bladder base (Figure 1). Additionally, scarring of the vagina and distal ureters was found and the patient suffered from a persistent MRSA-colonization of the urine. Due to the complex situation, we discussed an individualized treatment strategy with the off-label use of amniotic membrane (AM). Prior to the operation, AM was obtained immediately after elective cesarean sections with normal gestation. The donors were screened for infections including HIV, hepatitis and syphilis after informed consent. The placenta was cleaned with balanced salt solution and the amnion was separated from the chorion by blunt dissection under the laminar flow. The separated membranes were cut and after several rinsing steps the amnion was frozen at -20°C for 24 hours until further use. For further processing, the AM was defrosted in the water, sterilized in peracetic acid and alcohol mixture, then incubated for 2 hours on the shaker. After rinsing the amnion was applied on a sterile silicon scaffold and dried under laminar flow. Prior to surgery, three layers of dried AM were fixed together by a 4-0 Monocryl suture. During the surgery single-shot antibiotic was given and an extraperitoneal abdominal approach

Figure 1. Preoperative examination. A. Cystography showing the vaginal contrast leakage, indwelling ureteral stents. B. Cystoscopy showing the fistula canal at the bottom of the bladder.

Figure 2. Postoperative examination 3 months after the surgery. A. Cystography showing no contrast leakage, indwelling ureteral stents and catheter. B. Cystoscopy showing the amnion graft. AM merged with the surrounding bladder tissue at the margins but was still clearly demarcated with no signs of overgrowing urothelium or stroma. *, fixation points with suture.

was chosen to allow maximum exposure. The peritoneum was dissected off the bladder dome and posterior bladder wall. The incision was carried out through the anterior bladder wall to prepare the fistula. The ureteric orifices were found outside the fistula region. The fistulous tract and all devitalized tissue were excised sparingly. The multilayer AM of 4 cm size was used as a graft to close the defect and was fixed with 4-0 Monocryl sutures at the edges of the normal bladder wall. The anterior bladder wall was then closed and ureteric stents were led out in a suprapubic region to dry the bladder. Topical oestrogen was prescribed for 3 weeks after surgery. Anti-muscarinic therapy was started to restore the bladder capacity.

RESULTS

Seven days after the surgery a cystography was performed showing no leakage. In the three following months a reevaluation with cystography, cystoscopy and vaginal examination was performed with no signs of recurrent fistula and full recovery of the vaginal epithelium was presented (Figure 2). The ureter stents were internalized and the patient was able to micturate without incontinence. The bladder capacity was restored to the volume of 250 ml. No severe complications and no signs of graft rejection were observed during the follow-up of 6 months. The MRSA-colonization in the urine was successfully eradicated.

DISCUSSION

Complex fistulae are characterized by a size $>3\text{--}4\text{ cm}$, involvement of the urethra, presence of ureteric orifices, distinct vaginal scarring and multiple localization. They usually present a poor outcome with high

recurrence rates and a devastating course [1, 2, 6, 7]. Literature on the post-radiation fistulae is still scarce. Radiation-induced recurrent vesico-vaginal fistulae have the lowest success rate in terms of successful treatment and require the most demanding interventions [1]. The surgical approach to the repair of VVF depends on the surgeon's experience, the type and location of the fistula, and the patient's specific preferences [1, 2, 6]. The trans-vaginal approach is preferred in the case of distal fistulae and has been associated with lower morbidity rates. However, the abdominal or even laparoscopic approaches are favorable for deep and large fistulae. For the repair of this specific condition an interposition flap is needed to substitute the defect. The type of vascularized interposition flap is determined by how proximal (peritoneal, O'Connor approach, muscle flap) or distal (Martius labial fat) the genitourinary fistula is located in the vagina (85–100% success) [1]. The used flaps have the risk of infection, necrosis, loss of function after time and long-term morbidity. To avoid these problems different xeno-, homo- and autolog grafts have been proposed for the repair of VVF recently [1, 8]. Vascularization of the graft or flap is the main requirement for successful healing, otherwise the recurrence of fistulae occurs frequently. The AM is the innermost layer of the placenta and is being increasingly applied in reconstructive surgery and tissue engineering (wound healing, ophthalmology) due to its mechanical durability and "scaffolding" quality [9, 10]. The postoperative outcome after fistula repair significantly depends on the degree of angiogenesis and inflammation, which are regulated by the AM through cytokines (TGF- β , IL-10) and growth factors (VEGF) [10]. The AM also has an immunomodulatory effect, with no relevant tissue rejection being described in previous allogeneic or xenogeneic experiments [10, 11]. AM might also be used as an economically reasonable and alternative biomaterial for the treatment of VVF in developing countries with a high VVF incidence.

Introducing new surgical methods, innovation, as well as medical devices does not yet follow clear paradigms as is the case for the approval process of new drugs. The IDEAL procedure suggests and clearly provides stages, which allow to assign every method to its particular level of development and evidence. A new surgical method at the first "Idea level" should aim at proving a concept on a highly selected single-to-few patient basis. The suggested method of reporting should be a structured case report.

For the first time, we present a structured implementation of a new method for VVF repair following the IDEAL recommendations [5]. After positive results of this first case we plan consecutive prospective developmental studies in an animal model and cohort studies.

CONCLUSIONS

We reported a successful repair of VVF by an abdominal approach with an AM graft. Favorable results confirmed the technical simplicity, safety and efficacy of this procedure in the first case. Further research with controlled and randomized trials will show if a general application of the amnion graft to repair defects like VVF can be recommended.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Research Paper

Bladder Reconstruction with Human Amniotic Membrane in a Xenograft Rat Model: A Preclinical Study

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Received: 2016.10.28; Accepted: 2017.01.30; Published: 2017.03.11

Abstract

Background: Human amniotic membranes (HAMs) are assumed to have a number of unique characteristics including durability, hypoallergenic and anti-inflammatory properties.

Materials and Methods: Multilayer HAMs from caesarian sections were applied to repair defined bladder defects in male Sprague-Dawley rats. The animals were sacrificed at 7, 21 and 42 days after implantation. Bladder volume capacity after grafting was measured. Histological analyses were performed to assess a number of parameters including HAM degradation, inflammatory reaction, graft rejection and smooth muscle ingrowth.

Results: One rat died from sepsis in the treated group. No severe complications or signs of leakage were observed. Bladder capacity did not change over time. The initially increased inflammation in the HAM group diminished significantly over time ($p < 0.05$). No signs of HAM degradation were observed and smooth muscle staining increased over time.

Conclusions: HAMs appear to be durable and hypoallergenic grafts. The assumed suitability for the reconstruction of urinary tract justifies further research on detailed immunological process in larger grafts.

Key words: amniotic membrane, bladder augmentation, graft, rat experiment, IDEAL.

Introduction

Defects of urinary tract can be caused by a multitude of complications and events, such as catheterization-related urethral and ureteral strictures, surgery, stone passage or infections or fistulas. Preservation of continence and social autonomy after oncological surgery or radiation are gaining importance. One of the major reconstructive problems relates to lack of healthy native tissue for repair in patients, who have received multiple radiation treatments or undergone multiple surgeries. Autolog, xenolog and synthetic materials have been tested for the reconstruction of urinary tract defects,

but these experiments have not yielded any sustainable results for a number of reasons, including degradation, rejection or inflammation of the graft or scarring due to reduced perfusion.

New scaffolds and biomaterials are needed to achieve a fast regeneration without adverse effects. At present, four different approaches are applied: synthetic polymers (polyglycolic- (PGA), polylactic acid (PLA)), biologically derived materials (bladder acellular matrix), cell-based tissue engineering and composite materials [1]. Synthetic materials are available but cause additional complications and

serious concerns have recently been raised about the use of alloplastic materials in the pelvic floor [2]. The composite scaffolds were fabricated by binding collagen with PGA. Advances in tissue engineering technology have enabled seeding of scaffolds with autologous bladder epithelial and smooth muscle cells. Several experimental and clinical pilot studies have shown the potential of neo-bladder formation with a structure that is similar to that of native bladder tissue. For instance, improved bladder capacity was seen in seven patients with myelomeningocele following cystoplasty with engineered bladder tissue [3]. A prospective multicenter phase II trial from USA used a similar cell-seeded bio-degradable collagen scaffold for bladder augmentation in children and adolescents with spina bifida (n=11, follow up 36mos), but no improved bladder capacity or bladder compliance were observed, while several serious adverse events occurred [4]. Another strategy consists in applying naturally derived acellular matrices, such as small intestinal submucosa (SIS) or bladder acellular matrix (BAM), in an attempt to induce a native cell seeding of urothelial and smooth muscle cells from the neighboring native bladder tissue or ureters. Several experimental trials with rat, dog, sheep and porcine models have reported promising short-term results over the last two decades. However, the main problems are fibrosis and graft shrinkage of the large scaffolds, along with fast degradation, the need for immune therapy and the insufficient cell seeding due to a lack of tissue growth factors [5-9]. An Italian group augmented the bladders of five patients with exstrophic bladders using a SIS scaffold and included a long-term follow-up period of 3 years. After 6 months there was no histological evidence of SIS with feasible regeneration. However, poor results were obtained in terms of muscle regeneration, bladder capacity and continence [10]. Correct muscle alignment, proper innervation and vascularization are crucial for the development of larger contractile tissues that allow physiological voiding. Current research efforts in many centers are aimed at developing 'smart', biologically active biomaterials that improve tissue regeneration [11].

We hypothesized that the regeneration of important bladder wall components, such as urothelium, vascularization and smooth muscle, can be improved by application of hypoallergenic HAMs containing multiple growth factors. Outcomes of reconstruction of an experimental bladder defect using this scaffold were compared with outcomes in a group that was repaired with suture only. Following the evolving IDEAL-Device (Idea, Development, Evaluation, Assessment and Longterm) framework

for evaluation and regulation of surgical innovations, we established a preclinical xenograft model to provide evidence that HAMs are instrumental in repairing bladder defects. These experiments can be categorized as a stage 0 preclinical study for the evaluation of a new medical product according to IDEAL-D [12, 13].

Materials and Methods

Human amniotic membrane (HAM)

HAMs were obtained immediately after elective cesarean sections with normal gestation and informed consent of the patients. Ethical permission was obtained from the local ethics committee, University of Szeged. The study has been conducted according to the principles expressed in the Declaration of Helsinki. The donors were screened for infections including HIV, hepatitis and syphilis. The placenta was cleaned of blood clots with sterile phosphate-buffered saline (PBS) and the amnion was separated from the chorion by blunt dissection under the laminar flow. The separated membranes were cut into segments of at least 5 x 5 cm² with the epithelial side up. After several rinsing steps the HAMs were frozen at -20°C for 24 hours until further use. For further processing, the HAMs were defrosted in water, sterilized in peracetic acid and alcohol mixture, and incubated for 2 hours on the shaker. After rinsing the HAMs were prepared in four layers applied on a sterile silicon scaffold and dried under laminar flow (Fig. 1).

Experimental animals

The animal experiment was conducted at the Institute for Experimental Surgery of the University of Szeged, Hungary, in accordance with the National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals). The experimental protocol was approved by the Animal Welfare Committee at the University of Szeged (license number V./146/2013). The experiments were performed according to the EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and carried out in strict adherence to the NIH guidelines for the use of experimental animals. The study was approved by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority).

Twenty-seven male Sprague-Dawley rats, weighing from 320 to 380g and 3 months old, were housed and cared for at Szeged University's farm for experimental animal studies. All animals had free access to food and water and were cared for by an educated keeper and routinely inspected by a veterinarian.

Figure 1. A. Preparation of amniotic membrane with dissection from the chorion under laminar flow. **B.** Dried and cryopreserved multilayered amniotic membrane graft.

Operation procedure

The procedures were performed by two surgeons (DB and HG) using sterile surgical techniques. Sprague-Dawley rats were anesthetized with 40 mg/kg of ketamine 10%. The abdomen was shaved and prepared with an ethanol-propanol solution. Rats were operated in supine position. A microscope and microsurgical instruments were used. A midline laparotomy was performed. The bladder was identified and bladder capacity was determined (ml) before grafting in a standardized way. Evaluation of bladder pressure was performed via a cystometric method adapted from Lundbeck et al. [14]. After the dome of the bladder was exposed a 20 gauge needle was inserted through a small puncture in the dome of the bladder and secured in the place by a clamp. The bladder was emptied by a syringe. The needle was attached to an infusion system at 20 cm height above the bladder. Room temperature saline was infused by gravity and the bladder capacity was measured at the timepoint of infusion stop. The bladder was emptied, the procedure repeated two additional times, and the mean value recorded. Subsequently, the defined 0.5 cm lesion was cut at the bladder dome. In the treated amnion group (A, n=18) a multilayer amnion patch was trimmed overlapping the defect size (10x10 mm) and fixed to the bladder wall with 6-0 monocryl (Ethicon, Norderstedt, Germany) single sutures at three to four points. Additionally human fibrin glue (Evicel, OMRIX Biopharmaceuticals Ltd, Israel) was used to seal the lesion. In the first control group (C1, n=6) the defect was closed with a single monocryl 6-0 running suture and fibrin glue. In the second control group (C2, n=3) the amnion graft was sutured to the bladder wall without prior lesion. Fluid loss was compensated by administering 3 ml of 0.9% saline intraperitoneally at the end of surgery. Subsequently,

the abdominal fascia and skin were closed in layers with absorbable Vicryl 5-0 running suture and Monocryl 4-0 interrupted suture (Ethicon, Norderstedt, Germany).

The animals were sacrificed at one (A, n=5; C1, n=2; C2, n=1), three (A, n=6; C1, n=2; C2, n=1) and six (A, n=5; C1, n=2; C2, n=1) weeks after surgery. Bladder capacity was determined again. Subsequently, urinary bladder and tissue samples (urinary bladder, kidneys, spleen) were harvested and stored in 10% formalin solution for 2 hours and then stored in PBS at 4°C.

Clinical Assessment

Grafts and bladder wall were assessed regarding color, tissue contraction, inflammation and pliability by two surgeons (D.B. and H.G.). Results were documented by photograph.

Histology and Immunohistochemistry

All specimens were fixed and embedded in paraffin wax. Deparaffinized sections (5 µm) were used for staining with Hematoxylin and Eosin (H&E) to visualise tissue architecture and cell infiltration. Immunohistochemical staining was performed using an antibody against alpha-smooth muscle actin (α-actin; A2547; Sigma-Aldrich, Bornem, Belgium) to confirm the presence of smooth muscle fibres. Tissue sections were deparaffinized, rehydrated and subjected to a heat-induced antigen retrieval (Citrate Buffer, pH 6.0), followed by 3% hydrogen peroxide and avidin-biotin blocking. Prior to incubation with the primary antiserum, sections were incubated with PBS blocking solution containing 10% normal horse serum, 0.1% bovine serum albumin, 0.05% thimerosal, 0.01% NaN₃ and 1% Triton X-100. Primary and secondary antiserum were diluted in blocking solution without Triton X-100. Sections were

incubated overnight with the primary antibody and immunostained with streptavidin-biotin-peroxidase method, followed by diaminobenzidine (DAB) chromogen solution. Finally, sections were counterstained with hematoxylin and mounted in xylene-based mountant. Negative controls were incubated in blocking solution without primary antibodies. Pictures were taken using an Zeiss Axiophot microscope (Zeiss, Jena, Germany) equipped with an Olympus DP70 digital camera (Olympus, Münster, Germany) at 4x magnification.

Particular attention was paid to the slides showing the transition zone between the amnion graft and normal bladder wall. Semi-quantitative scores from 0 to 3 for inflammation were used. Inflammation of the implant region was scored by counting lymphocytes in 10 fields of 0.25 mm² in three observer-randomized H&E slides (semi-quantitative score: 0 = <5% cells/field; 1 = 5-25%; 2 = 25-50%; 3 = >50%; 200x magnification). AM-thickness was measured in µm to assess degradation and inflammation. Kidneys and spleen specimen were analysed for signs of transplant rejection.

Statistical analysis

The data were documented into Microsoft Excel software and then transferred into a GraphPadPrism6.0 (Graphpad Software, Inc.) data bank for statistical analysis. Continuous data were checked for normality of distribution before choosing between parametric and non-parametric tests. The results were presented as medians with range or means with standard deviation (SD) in case of normal distribution. Data from different groups were compared using the Mann-Whitney U test and the Kruskal-Wallis test. Statistical significance was assumed at $p < 0.05$.

Results

Clinical course and functional results

Two animals (11%) died in the treated group (A), one animal due to postoperative sepsis and another animal during anesthesia, respectively. No animals from the control groups C1 and C2 died. No other severe complications higher than grade II (Clavien-Dindo classification) were observed. The bladder capacity did not change in the treated group A but reduced significantly in the control group C1 with suture of the lesion ($p=0.01$) (Fig. 2).

Macroscopic examination

7 days

No signs of severe inflammation were found in the abdominal cavity during resurgery.

Meso-adhesions to the HAM graft were detected in most treated cases. The multilayer amnion was clearly detectable as a shiny, reddish layer on the bladder wall. HAM appeared as a thick, edematous graft with inflammation running from the middle towards the transition zone of the bladder wall. Blister formation was seen in one case. Inflammation was less prominent in the control groups (C1 and C2).

Figure 2. Bladder capacity pre- and postoperatively in the amnion treated group A and control group C1. The bladder capacity reduced significantly in the control group due to the scarring of the sutured lesion (* $p < 0.05$).

21 days

HAM was still well defined, albeit with reduced inflammation. The edema had almost disappeared and the amnion graft appeared as a reddish layer on the bladder wall (Fig. 3). Adhesions were still present in some cases.

42 days

HAM was still recognizable, but the colour had changed to a whitish-grey with signs of inflammation and blood vessels formation in the transition zone of the bladder wall (Fig. 3).

Microscopic examination

7 days

The xenotransplanted HAM graft covered the bladder wall and maintained its architecture in the treated group. The lesions could be recognized as the regions without smooth muscle cells but with abundant connective tissue and signs of inflammation. Significant inflammation and increased blood vessels numbers were observed in the amnion and between the amnion and the adventitia of the bladder, which resulted in an enlarged amnion.

Infiltrated lymphocytes agglomerated mostly in the area bordering the HAM. No shrinkage of the transplanted grafts was seen in any of the specimens.

Lower inflammation levels were found in the control groups C1 and C2 (Fig. 4).

Figure 3. Macroscopic and histological evaluation (H&E staining) of inflammation and AM degradation over time (21 and 42 days after grafting). Reduction of inflammation over time. Decreased inflammatory cells (*) and increased vascularization (V) in the periamniotic transition zone were detected. No signs of AM degradation. Scale bar 200µm.

Figure 4. Reduction of inflammation over time, semiquantitative analyses of inflammatory cells: Treated (A): Amnion repair of bladder defect. Control/Lesion (C1): Closure of bladder defect with suture. Control/Amnion (C2): Amnion bladder onlay without defect. Semi-quantitative score: 0 = <5% cells/field; 1 = 5-25%; 2 = 25-50%; 3 = >50%; 200x magnification. Data is expressed as median with range. *, significant reduction (p<0.05).

Figure 5. Measurement of amnion thickness over time (7, 21 and 42 days): Treated (A): Amnion repair of bladder defect. Control/Amnion (C2): Amnion bladder onlay without defect. Data is expressed as mean with SD, p<0.05.

Figure 6. Immunohistological analysis of a reconstructed urinary bladder wall at timepoint 3 (42 days). A: Amnion group, reconstruction with AM graft; B: Control group, reconstruction with suture, AM; Amniotic membrane, Ur; Urothelium, BL; lumen of urinary bladder, X; region of lesion in the bladder wall. (A) H&E staining displaying mild inflammatory infiltration. α -Actin staining reveals frequently arranged smooth muscle bundles (arrows). Strong immunoreactivity underneath the urothelium layer is observed. The region of the lesion can be clearly recognized in the control group with muscle bundles, while there is a smooth muscle ingrowth in the treated group.

21 days

The amnion including its different layers was mostly still clearly recognizable. No signs of amnion degradation or necrosis were observed, but the amnion appeared less thick compared to time point 1 (Fig. 5). Inflammation of the amnion was significantly diminished ($p < 0.05$) (Fig. 3, Fig. 4 and Fig. 6). However, a moderate number of lymphocytes and a few eosinophils were detected in the transition zone between the graft and the surrounding tissue. New capillaries started to grow into the surrounding connective tissues and scattered smooth muscle cells appeared in the area of the lesion. In the control groups C1 and C2 signs of inflammations (presence of lymphocytes) had mostly disappeared and the differences were less obvious. More connective tissue with scattered smooth muscle cells was discerned (Fig. 3).

42 days

It has become more difficult to discriminate the region of the lesion in the treated group, where the amnion formed a thinner layer on the bladder wall with no signs of degradation (Figures 3 and 5). Inflammation was markedly reduced in the amnion and in the zone between the amnion and the bladder wall ($p < 0.05$) (Figures 3 and 4). The number of large vessels in the amnion appeared to be reduced and

periamniotic vascularization increased but these results were not significant. Connective tissue, bundles and thin muscle layers were abundantly found in all groups. Smooth muscle regeneration appeared faster in the treated group (Fig. 6), although the difference in the regeneration compared to the control group with suture of the lesion was not significant ($p < 0.05$).

Rejection

No macroscopic signs of rejection were found in the kidney or spleen specimens. However, 4 out of 6 animals in the treated group showed an affected kidney at time point 2. The changes were subtle with slightly enlarged tubuli and urinary space. The glomeruli appeared denser. No immune cells or other signs of transplant rejection were observed. At time point 3, only the kidneys of 2 of the 5 animals were still slightly affected, while no such alterations were detected in the controls without amnion had no such alterations. To exclude an obstruction or increased bladder pressure due to the HAM graft as the cause for these findings, we compared the results with another study, where we used amnion graft on colon (unpublished data). The same alterations were detected in the kidneys in this group. Taken together, a subtle, transient transplant glomerulitis is suggested in case of HAM graft in a rat.

Discussion

The aim of our study was to assess the functional, inflammatory and allergenic characteristics of human amniotic membrane in a xenograft rat model.

HAMs have been widely used for decades in ophthalmic surgery, mainly to cover defects after corneal ulcerations [15, 16]. Today, HAM grafts are considered the standard therapy to reconstruct the eye surface as shown in several randomized and controlled trials [17, 18]. The effectiveness of HAMs has been amply demonstrated in several clinical studies, for instance, as skin graft donor site dressing in burn patients or for the reconstruction of dental defects and oro-pharyngeal fistulas [19-22]. Furthermore, tissue-engineered HAMs have been used as matrix for cell seeding and expansion of epithelial progenitor cells in ophthalmology, orthopedics, healing of liver dysfunction, etc. [23, 24].

Currently there is a growing interest in extending the application possibilities of HAMs due to their wide availability, low costs and interesting properties. The technique of applying HAMs in reconstructive urology was introduced for the first time in 1955 although only a few groups applied AM to repair the urinary tract wall [25]. Reconstruction of urethrae with HAMs in a rabbit model showed a proper re-epithelialization, and even better results could be achieved with a denuded human amniotic scaffold inoculated with primary rabbit urethral epithelial cells [26, 27]. A Polish group described a technique to supplement long ureteral wall strictures (5.5 cm) by using folded HAM allografts and presented good, sustainable results after an average follow-up period of 25.2 months [28]. Brandt and coworkers successfully reconstructed a female urethra using autologous grafts prepared from HAMs [29]. Reconstruction of a complex vesico-vaginal fistula with a HAM interposition patch was shown in two case reports [30, 31]. Excellent integration of the implanted amnion graft within the host urinary tract wall and reduced fibrosis were reported after the reconstructive procedures. Adamowicz and coworkers designed a sandwich-structured biocomposite material from a frozen cell-seeded (bone marrow-derived mesenchymal stem cells) HAM and covered it on both sides with two-layered membranes prepared from electrospun poly-(L-lactide-co-caprolactone) (PLCL). The authors considered this reinforcement of the AM necessary because of its poor mechanical qualities. The new biomaterial (10x10 mm) was used for bladder augmentation after hemicystectomy in 10 rats, which were sacrificed after 3 months [32]. Immunohistochemical analysis revealed effective regeneration of the urothelial and smooth

muscle cells and complete PLCL degradation. However, the authors reported a moderate inflammatory reaction after 3 months.

In contrast, the results of our study confirm previous reports in which the elasticity and durability of multilayer HAMs were described and no signs of leakage and unchanged bladder capacity were observed after the reconstruction. A modified "simple cystometry" by infusion of flood by gravity at a pressure head of 20cm was used in our experiments, sensitivity comparable to standard multichannel cystometry [33]. The cystometric results were highly reproducible on repeated assessments, confirming the reliability of this procedure to assess the bladder capacity in the rat model. The differences in bladder volume change were only about <15% of the total volume, due to a small defect. More obvious reduction of bladder capacity is known from patients with partial bladder resection with the need for augmentation surgery. Future studies with larger grafts are planned.

The inflammatory reaction had almost disappeared already after 6 weeks. The mechanical behavior of the urinary bladder is dependent on the properties of the extracellular matrix and smooth muscle cells. The extracellular matrix (ECM) of HAMs is composed of collagen (type I, III, IV, V and VI), fibronectin, nidogen, laminin, proteoglycans and hyaluronan in a proportion that is similar to the basement membrane of urinary tracts [34]. With a diameter of about 150 - 200 nm amniotic membrane is one of the thickest membranes of the human body and its stroma provides elasticity and mechanical strain. Large grafts need sufficient nutrition of the cells and removal of waste products to eliminate/reduce the risk of fibrosis and shrinkage. Having a diffusion distance from the supplying blood vessel of ~150-200 µm, HAMs efficiently conduct sufficient oxygenation of cells by diffusion [35]. No signs of graft shrinkage or necrosis were found during the 6-wk follow-up period.

The limitations of our study were the small size of the grafts (10x10mm), missing detailed data on immunological process of regeneration and the short follow-up period. Regeneration in large constructs or even neo-bladder needs further evaluation. Multiple soluble active growth factors have been identified within cryopreserved HAMs [36]. This naturally derived composition of incorporated growth factors is predisposed to support fetal healing - rapidly and without an inflammatory response - resulting in a complete restitution of normal tissue function [37]. HAMs secrete the glycoprotein lumican and growth factors like epidermal growth factor (EGF), a hepatocyte growth factor (HGF) and keratinocyte

growth factor (KGF), which stimulate epithelial cell growth [36]. Wound healing is a highly complex process of inflammation, regeneration of tissue, angiogenesis, scarring and reepithelialization. Amnion was previously described to reduce the scar tissue building by the inhibition of fibroblasts. There is no scarring after the child injuries in the amniotic sac. Kim and Tseng transplanted HAM for surface reconstruction of cornea in vitro and in animal studies [15]. They detected reduced fibroblast building by suppression of Transforming growth factor- β (TGF- β). Further studies confirmed that amniotic stroma includes factors which inhibit the proliferation of myofibroblasts [38]. The results of the presented study support the hypothesis of faster tissue regeneration by inhibition of scar building. However, a detailed immunohistological investigation of regeneration process in larger grafts would be a part of the future work to be done. Although we expected a faster regeneration of smooth muscle in the amnion group due to the presence of growth factors, our results were not significant in this respect and it was not possible to compare the regeneration in a standardized way due to the small size of the grafts used and an overlap with the normal bladder wall.

The overall low complication rate was comparable to previous reports [26-32]. One animal in the treated group (5.6%) died due to sepsis. All animals in the amnion group presented a transient local inflammation and the amnion group included several cases of local abdominal adhesions, but no signs of peritonitis. Akle and Adolfini detected no signs of acute rejection after allogeneic subcutaneously implanted HAMs [39]. Fas ligand positive cells, suppression of TGF- β are other examples for immunological privilege of the amniotic membrane. The marked edema in the first week and high inflammation score in the amnion graft group may reflect the local immunological rejection in the presented study. However, this reaction is transient and no differences between the groups were found in follow up. A similar transient increase in inflammatory cells was reported by Kesting et al., who used cryopreserved HAMs for soft tissue repair in rats [40]. Additionally, most animals of the amnion group presented a subtle acellular transplant glomerulitis. These changes can be classified as borderline mild acute rejection according to the Banff classification [41]. However, due to the relatively short follow-up period, it was not possible to determine whether chronic glomerulitis with interstitial fibrosis and tubular atrophy would develop. Still, 2 of 5 animals were slightly affected in the amnion group. We suggest that the glomerulitis observed during our experiments was due to the

xenolog model and that glomerulitis would normally not develop in an allogeneic human setting. However, further studies on immunological process are needed to confirm this assumption.

We suggest that HAMs are appropriate for the reconstruction of small defects (fistula grafting, urethro- and ureteroplasty), but not of the whole organ, which requires healthy tissue ingrowth. Experiments with larger scaffolds need to be conducted to further explore the clinical potential of HAMs. Following the IDEAL-D recommendations, further evaluation of possible indications in clinical studies is needed after successful stage 0 experimental results.

Conclusions

Preclinical xenotransplantation model supports the application of HAMs for reconstruction of urinary tract. Successful closure of defined bladder wall defects could be achieved by using multilayered HAMs. The inflammation and rejection were subtle and transient and smooth muscle ingrowth appeared to be enhanced. These findings need further support by research in larger animals and in human allogeneic settings to explore immunological HAM impact and possible further applications of HAMs in reconstructive surgery.

Abbreviations

BAM: bladder acellular matrix; DAB: diaminobenzidine; ECM: extracellular matrix; EGF: epidermal growth factor; IDEAL: idea, development, evaluation, assessment and longterm evaluation; ITERA: International Tissue Engineering Research Association; ITERM: Institute of Tissue Engineering and Regenerative Medicine; HAM: human amniotic membrane; HGF: hepatocyte growth factor; KGF: keratinocyte growth factor; PBS: phosphate-buffered saline; PGA: polyglycolic acid; PLA: polylactic acid; SIS: small intestinal submucosa; PLCL: poly-L-lactide-co- ϵ -caprolactone; TGF- β : transforming growth factor- β .

Acknowledgements

The authors highly appreciate the outstanding support throughout the experiments from Albert Ramon, International Tissue Engineering Research Association (ITERA), Belgium and Peter Ponsaerts, Laboratory of Experimental Hematology, University of Antwerp, Belgium. Special thanks for the laboratory support go to Annette Wiggen-Kremer, Institute of Tissue Engineering and Regenerative Medicine (ITERM), Lukas Hospital Neuss, Germany.

Competing Interests

The authors have declared that no competing interest exists.

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Human Amniotic Membrane Is Not Suitable for the Grafting of Colon Lesions and Prevention of Adhesions in a Xenograft Rat Model

Surgical Innovation

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DOI: 10.1177/1553350617709828

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Abstract

Introduction. New biological materials are needed for specific applications in reconstructive bowel surgery and for the prevention of adhesion formation. Amniotic membranes (AMs) are assumed to have a number of unique characteristics that enhance the ingrowth of the surrounding tissue. The aim of the present study was to provide proof of these qualities in a xenograft model. **Materials and methods.** A multilayer human AM (HAM) was applied to repair defined colon wall defects in Sprague-Dawley rats ($n = 18$). The control group was repaired with a suture ($n = 6$). The animals were killed humanely at 7, 21, and 42 days after implantation. Adhesions and perioperative complications were examined. Histological and immunohistological analyses were performed to assess a number of parameters, including degradation of the HAM, inflammation, graft rejection, and smooth muscle ingrowth. **Results.** Two rats in the treated group died. No other severe complications were observed. Adhesion formation was more prominently visible in the HAM group ($P < .05$). The initially increased inflammation in the HAM group reduced over time but remained significantly increased ($P < .05$). The HAM degraded over time and a subtle transient glomerulitis could be observed. **Conclusion.** HAMs were found to increase adhesion formation and were not suitable for bowel augmentation in the presented xenograft model.

Keywords

amniotic membrane, xenolog transplantation, bowel lesion, anastomosis, adhesions, IDEAL recommendations

Introduction

Anastomotic leakage (AL) is a major complication of bowel reconstructive surgery, occurring in 4.8% to 37.5% of cases.^{1–3} AL causes consecutive peritonitis, adhesions, and fistula formation with significant morbidity and mortality. Adhesion formation is a serious issue of bowel surgery and has been found to be highly prevalent in patients with a history of multiple abdominal operations or peritonitis. These patients are at a high risk of development serious intraoperative complications during a subsequent operation if adhesiolysis is performed. These complications include bowel perforation, ureteral or bladder injury, and vascular injury.^{4,5} Much research efforts have been devoted to finding materials and improved surgical techniques to protect high-risk gastrointestinal anastomosis during the critical days of healing. Allogeneic (peritoneum), xenolog (collagen), and synthetic materials (polymers) and sealants

(fibrin) have been previously tested to protect the anastomosis and prevent AL and adherence formation.^{6–10} Most of the trials were animal trials and have not yet been able to identify the optimal barrier to prevent adhesions in a sustainable way.¹¹ In most trials, detailed information on materials and methods according to IDEAL criteria is missing.¹² Synthetic materials, such as polymers, are available but give rise to additional complications, and serious concerns

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have recently been expressed about the use of alloplastic materials.^{8,13}

We hypothesized that application of hypoallergenic human amniotic membranes (HAMs) containing multiple growth factors could improve the regeneration of the colon wall and prevent adhesions caused by their anti-inflammatory characteristics. Outcomes of reconstruction of an experimental colon wall defect using a HAM graft were compared with outcomes in a group that was repaired with suture only. Mechanical characteristics, such as dehiscence and adhesion formation, were examined, and a number of parameters, including AM degradation, inflammation, possible rejection, and tissue ingrowth, were histologically analyzed. Following the IDEAL-D system of surgical innovations, we established a preclinical xenograft model in an attempt to provide evidence that HAMs are instrumental in repairing rat bowel defects. These experiments can be categorized as a stage 0 preclinical study.^{12,14}

Materials and Methods

Human Amniotic Membrane

HAMs were obtained immediately after elective cesarean sections with normal gestation. The donors were screened for infections, including HIV, hepatitis, and syphilis, after informed consent. The placenta was cleaned of blood clots with sterile phosphate-buffered saline (PBS), and the amnion was separated from the chorion by blunt dissection under laminar flow conditions. The separated membranes were cut into blocks of at least $5 \times 5 \text{ cm}^2$ with the epithelial side up. After several rinsing steps, the HAMs were frozen at -20°C for 24 hours until further use. For further processing, the HAMs were defrosted in water, sterilized in peracetic acid and alcohol mixture, and then incubated for 2 hours on the shaker. After rinsing, the amnion was prepared in 4 layers applied on a sterile silicon scaffold in order to hold shape and dried under laminar flow.

Experimental Animals

The animal experiment was conducted at the Institute for Experimental Surgery of the University of Szeged, Hungary, in accordance with the National Institutes of Health (NIH) guidelines (Guide for the Care and Use of Laboratory Animals). The experimental protocol was approved by the Animal Welfare Committee at the University of Szeged (license number V./146/2013). The experiments were performed according to the EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and carried out in strict adherence to the NIH guidelines for the use of experimental animals. The study

was approved by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority).

A total of 27 male Sprague Dawley rats, weighing between 320 and 380 g and 3 months old, were housed and cared for at Szeged University's farm for experimental animal studies. All animals had free access to food and water and were cared for by an educated keeper and routinely inspected by a veterinarian.

Operation Procedure

The procedures were performed by 2 surgeons (DB and HG) using sterile surgical techniques. The 27 adult male Sprague-Dawley rats were anesthetized by intraperitoneal injection of 40 mg/kg of ketamine 10%. The abdomen was shaved and prepared with an ethanol-propanol solution. Rats were operated in the supine position. A microscope and microsurgical instruments were used. A midline laparotomy was performed under antiseptic conditions. The colon was identified and a defined 0.5 cm of the cecum wall was resected. In the treated amnion group (A, $n = 18$), a multilayer amnion patch was trimmed overlapping the defect size ($10 \times 10 \text{ mm}^2$) and fixed to the colonic wall with 3 or 4 interrupted 6-0 Monocryl sutures (Ethicon, Norderstedt, Germany). Additionally, human fibrin glue (Evicel, OMRIX Biopharmaceuticals LTD, Israel) was used to seal the lesion. In the first control group (C1, $n = 6$), the defect was closed with a single Monocryl 6-0 running suture and fibrin glue. In the second control group (C2, $n = 3$), the amnion graft was sutured to the colonic wall without prior lesion. Fluid loss was compensated by administering 3 mL of 0.9% saline intraperitoneally at the end of surgery. The abdominal muscle layer and skin were closed separately with absorbable Vicryl 5-0 running sutures and Monocryl 4-0 interrupted sutures (Ethicon). Animals were fed with standard rat chow and water starting at the sixth hour postlaparotomy.

The animals were killed humanely at 1 (A, $n = 5$; C1, $n = 2$; C2, $n = 1$), 3 (A, $n = 6$; C1, $n = 2$; C2, $n = 1$), and 6 (A, $n = 5$; C1, $n = 2$; C2, $n = 1$) weeks after surgery. Subsequently, tissue samples (colon, kidneys, spleen) were harvested and stored in 10% formalin solution for 2 hours and then stored in PBS at 4°C .

Clinical Assessment

During postmortem examination, the grafts were examined macroscopically by 2 surgeons (DB and HG). Existence of peritonitis, and abscess and adhesion formation were recorded. The colon wall was assessed regarding adhesions and inflammation. The adhesion-covered area was graded semiquantitatively between 0 and 3 according to the score developed by van der Hamm et al.¹⁵ (Table 1). Results were documented by photographs. After

Table 1. Adhesion Grading Scale.¹⁵

0	No adhesions
1	Minimal adhesions, mainly between the omentum and the bowel graft
2	Moderate adhesions, that is, between the bowel graft and the omentum or a loop of the small bowel or the abdominal wall
3	Severe and extensive adhesions, that is, between the bowel graft and several loops of small bowel and the abdominal wall, including abscess formation

macroscopic assessment, the grafted or sutured regions were excised with at least a 2-cm rim of surrounding tissue. The complications were examined according to Clavien-Dindo classification.¹⁶

Histology and Immunohistochemistry

The histological examination was performed by Laboratory of Cell Biology and Histology, University of Antwerp, Belgium. All specimens were fixed and embedded in paraffin wax. Deparaffinized sections (5 μ m) were used for staining with hematoxylin and eosin (H&E) to visualize tissue architecture and cell infiltration. Particular attention was paid to the slides of the transition zone between the amnion graft and normal colon wall. A semiquantitative score from 0 to 3 for inflammation and from 0 to 2 for vascularization were used. Inflammation of the implant region was scored by counting lymphocytes in 10 fields of 0.25 mm² in 3 observer-randomized H&E slides (semiquantitative score: 0 = <5% cells/field; 1 = 5%-25%; 2 = 25%-50%; 3 = >50%; 200 $\times$ magnification). A similar score was applied for vascularization (0 = 0 vessels/mm²; 1 = 1-3 vessels/mm²; 2 = >3 vessels/mm²; 200 $\times$ magnification). AM thickness was measured in micrometers to assess degradation and inflammation. Immunohistochemical staining was performed using anti-smooth muscle actin (α -actin; A2547; Sigma-Aldrich, Bornem, Belgium) to confirm the presence of smooth muscle fibres. After deparaffinization and a heat-induced antigen retrieval, the slides were incubated with the primary antibody of the corresponding antigen. The antibody was visualized using a streptavidin-biotin-peroxidase method, followed by diaminobenzidine chromogen solution. Digital images of H&E and α -actin were used for the evaluation of smooth muscle content within the reconstructed colon wall.

Statistical Analysis

Qualitative variables were presented as frequencies and percentages. Quantitative variables were expressed as means $\pm$ SDs. Data from different groups were compared using the Mann-Whitney *U* test and 2-way ANOVA,

respectively. Statistical significance was assumed at $P < .05$. Statistical analysis was performed using the GraphPadPrism 6.0 statistical software package.

Results

Clinical Course and Functional Results

Two animals (11%) died in the treated group (A)—1 animal as a result of postoperative sepsis and 1 during the anesthesia—and were hence excluded from analysis. No animal from the control groups C1 and C2 died. No other severe complications higher than grade II (Clavien-Dindo classification) were observed.

Macroscopic Examination

At 7 Days. No signs of severe inflammation were found in the abdominal cavity during resurgery. Strong adhesions of the HAM graft to the small bowel and abdominal wall that withstood tractions were detected in most treated cases. In 2 of 5 cases, there was a blister and edema formation (Figure 1). The average adhesion formation differed significantly among groups. Specifically, a higher adhesion score with a larger coverage area was found in the amnion group A (1.8 ± 0.45) versus the C1 group (0.5 ± 0.7 ; $P < .05$; Figure 2). However, similar adhesion scores were detected for C1 and C2. The AM appeared as a thick edematous graft infiltrated by inflammation signs. The inflammation was less present in the control groups (C1 and C2).

At 21 Days. The HAM was still well defined, albeit with reduced inflammation. The edema and adhesions were less conspicuous, and the amnion graft appeared as a thinner reddish layer on the colon wall. The adhesion score was higher in the amnion group A (1.8 ± 0.84) when compared with the C1 group (1 ± 0 ; $P = .178$) but did not reach the significance level (Figure 2).

At 42 Days. The HAM was hardly recognizable and in some cases not detectable. The control group C2 was characterized by the absence of the HAM, which had been completely degraded. The signs of inflammation reduced; however, they were still stronger in the amnion group A. The adhesions increased in the amnion group when compared with the C1 and C2 groups ($P = .052$; Figures 1 and 2).

Microscopic Examination

At 7 Days. The xenotransplanted HAM graft covered the colon wall and maintained its architecture in the treated group. The lesions could be recognized as the regions that

Figure 1. Macroscopic evaluation of adhesion score in the amnion treated group A (Table 1). A. Strong adhesions to small bowel and abdominal wall with edema and inflammation of the lesion site were detected at time point T1 (7 days). B. Moderate adhesions without signs of inflammation were detected at time point T3 (42 days).

Figure 2. Adhesion formation in the 3 groups: Group A, grafting of lesion with amnion; group C1, closure of colon lesion with suture; group C2, amnion onlay without lesion. Time point 1: 7 days; time point 2: 21 days; and time point 3: 42 days. Means $\pm$ standard errors are plotted. Overall comparisons with 2-way ANOVA test ($P < .05$) for time points 7, 21, and 42 days. A higher adhesion score was found in the amnion group A versus the C1 group ($P < .05$ for time point 1) and slight increase of adhesion in the amnion group over time.

Abbreviation: HAM, human amniotic membrane.

lacked smooth muscle cells. Strong inflammation with abundant increased numbers of lymphocytes and blood vessels was observed in the amnion layers and between the amnion and the submucosa of colon, which resulted

in an enlarged amnion. A lower but still strong inflammation was found in the control groups C1 and C2 as well (Figures 3 and 4).

At 21 Days. The amnion, including its different layers, was mostly still clearly recognizable. Its thickness reduced (Figure 5), and inflammation was significantly diminished ($P < .05$; Figures 3 and 4). No change in vascularization was observed compared with time point 1 (data not shown). Connective tissue bundles and scattered smooth muscle cells appeared in the area of the lesion (Figure 3). In the control group C1, signs of inflammation (presence of lymphocytes) had mostly disappeared, and there were also no clear signs of regeneration of smooth cells in the lesion region.

At 42 Days. It became more difficult to verify the presence of the amnion in the treated group; the different layers of the amnion could no longer be distinguished, and the amnion, which was significantly reduced in thickness, formed a thinner layer on the colon wall (Figures 3 and 5). In the control group without lesion (C2), the amnion was completely degraded and could no longer be detected. Inflammation was markedly reduced in the amnion and in the zone between the amnion and the colon wall but was still significantly higher compared with the control group C1 ($P < .05$; Figures 3 and 4). Despite the presence of scattered smooth muscle cells and bundles, it was difficult to measure if there was a clear regeneration of smooth muscle compared with the control group C1 (Figure 3).

Rejection

No macroscopic signs of rejection were found in the kidney and spleen specimens. However, 4 out of 6 animals in the treated group showed an affected kidney at time point 2. The changes were subtle, with slightly enlarged tubuli

Figure 3. Histological evaluation of AM degradation and inflammation in the treated group over time (A: 7 and B: 42 days after grafting). Arrows show the AM thickness. Significant AM degradation and reduction of inflammation over time can be seen. Decreased inflammatory cells and increased vascularization in the periamniotic transition zone were detected. Scale bar 200 μ m. Abbreviations: AM, amniotic membrane; Muc, colon mucosa.

Figure 4. Reduction of inflammation over time, semiquantitative analyses of inflammatory cells: means $\pm$ standard errors are plotted. T1: 7 days; T2: 21 days; T3: 42 days. Group A, grafting of lesion with amnion; group C1, closure of colon lesion with suture; group C2, amnion onlay without lesion. Semiquantitative score: 0 = <5% cells/field; 1 = 5%-25%; 2 = 25%-50%; 3 = >50%; 200 $\times$ magnification. A significant decrease of inflammation in the treated group T2 versus T1 was detected ($P < .05$). However, the presence of inflammatory cells was significantly higher in the treated versus control/lesion group at every time point ($P < .05$). Abbreviation: HAM, human amniotic membrane.

and urinary space. The glomeruli appeared more dense. No presence of immune cells or other signs of transplant rejection were found. At time point 3, only the kidneys of 2 out of 5 animals were still slightly affected, whereas no

Figure 5. Thickness of the multilayer amniotic membrane in micrometers: means $\pm$ standard errors are plotted. T1: 7 days; T2: 21 days; T3: 42 days. Group A, amnion graft repair of colon lesion; group C2, amnion onlay without lesion. A significant reduction of human amniotic membrane (HAM) thickness was seen over time T3 versus T1 in both groups ($P < .05$).

such changes were detected in the controls without amnion. Taken together, a transient, subtle transplant glomerulitis is suggested in the rat HAM grafts.

Discussion

The HAM has several characteristics that make it ideal for use as biological material in surgical interventions. Since their introduction in the 1990s as a promising

cryopreservation method with interesting long-term storage possibilities,¹⁷ HAMs have been broadly applied in a wide range of clinical studies as corneal replacement, skin graft donor-side dressing in burn patients, and graft for oropharyngeal fistula.¹⁷⁻²¹ Currently, there is a growing interest in extending the application possibilities of HAMs because of their wide availability, low costs, and biological properties.

The aim of our study was to assess the mechanical, inflammatory and allergenic properties of HAMs in a xenograft rat model in the hope that successful xenotransplantation might pave the way for clinical applications according to the IDEAL stages of surgical innovation.¹² Here, we present a preclinical stage 0 animal study according to the IDEAL-D system.¹⁴ However, the present study demonstrated that the use of AMs for repair of bowel lesions in a xenograft model is not beneficial compared with standard therapy. Moreover, the incidence of adhesion formation (89% in the treated group vs 33% in the control group, $P < .05$) and inflammatory reactions was significantly higher in the amnion group. However, HAM proper seems to have no antigenic effect, as also corroborated by the results of a human allogeneic experiment showing no acute transplant rejection of subcutaneously implanted HAMs.²² Although we did not find any clinical signs of rejection in our xenolog experiment, we did observe extended adhesions and an inflammatory reaction in the amnion. Similar increased number of inflammatory cells were reported by other groups.^{18,23,24} Additionally, most animals of the amnion group presented a subtle acellular transplant glomerulitis. The changes can be classified as borderline mild acute rejection according to the Banff classification.²⁵ However, because of the limited 6-week follow-up period, it was not possible to assess whether chronic glomerulitis with interstitial fibrosis and tubular atrophy would occur.

The formation of adhesions is a frequently observed process following surgical interventions in the peritoneal cavity.^{11,26} It is now well accepted that the inflammatory system plays an important role in the regulation of both the coagulation and fibrinolytic systems, which are crucial for the genesis of adhesions.²⁶ Several strategies for the prevention of adhesions have been proposed, including inhibition of inflammation, prevention of fibrin formation, promotion of fibrinolysis, and antiangiogenesis and tissue engineering.^{6,27-29} We assumed that the HAM's anti-inflammatory properties would reduce the formation of adhesions. For this purpose, we decided to use the epithelial side of the HAM facing the abdominal cavity.²⁴ However, our results showed that the adhesions could not be prevented and were even increased in the amnion group, probably as a result of increased inflammation by insufficient bowel sealing with amnion graft and rejection in the xenograft model.

Adequate bowel perfusion is a key determinant of successful healing.³⁰ The nutrition of amnion is ensured by diffusion. Additionally, HAMs contain growth factors that promote epithelial wound healing. Although we expected faster regeneration of the "neo-colon" wall in the amnion group because of growth factors, our results were not significant, and it was not possible to compare the regeneration in a standardized way because of the small size of the grafts and an overlap with the normal colon wall.

Only a few groups have applied HAMs in reconstructive bowel surgery to date. Schmidt et al²⁴ used a patch of monolayer HAM to repair an 8-mm duodenal lesion in 42 Wistar rats. The animals were followed up for 28 days, and adhesions were detected in all animals. Two animals presented with obstruction, and 1 animal died from peritonitis. The HAMs degraded after 14 days, and the regeneration of mucosa and smooth muscle increased after time. Epithelialization of the HAMs started 3 days after surgery and was completed between 3 and 4 weeks. The authors concluded that HAMs can be used as a temporary seal to reestablish the duodenal wall structure. The colon bacteria digest the amnion tissue and cause the degradation of the patch. Several studies could show the degradation of the extracellular matrix components by bacterial-derived metalloproteases in colon.^{31,32} If applied as a graft, a fast degradation of amnion is reported between 14 and 90 days, depending on the grafted tissue and number of layers.^{18,24} In the case of monolayer amnion, the degradation is almost completed 2 weeks postimplantation. Transformation of multilayer HAMs and tissue reorganization and degradation were observed between 21 and 42 days in our experiment. In our study, additionally, fibrin glue was used to seal the lesions in all groups. No adverse effects of fibrin glue on adhesion formation were reported previously.³³

Using a peritonitis rat model, a Turkish group covered a cecum anastomosis with a 10-mm amnion wrap. The AL rate amounted to up to 25% in the standard anastomosis group versus 0% in the amnion group after a 7-day follow-up period. Neoangiogenesis, fibroblast activity, and collagen deposition were significantly higher in the groups with AMs ($P < .05$).^{23,34} Unlike in our model, the authors reported a high dehiscence rate of 40% to 50% in the control group with standard anastomosis; this is probably the result of a different experimental model used.²³ In our study, no signs of dehiscence were detected in the C1 group with suture of a small bowel wall defect. However, the increased initial inflammation and adhesions in the amnion group appear to be signs of insufficient bowel sealing with amnion graft. Additionally, 1 case of postoperative peritonitis and sepsis was detected in the HAM group. Another study used light-activated HAM wrap to strengthen colonic anastomosis and reduce perianastomotic adhesions in Sprague-Dawley rats.³⁵ A

1-cm wide strip of HAM (stromal side down) was wrapped around the anastomotic line and sealed to the serosal surface by illumination with 532 nm light. The illumination provided photochemical bonding and better sealing of HAM with less AL and less inflammatory reaction, unlike in our experiments.

There were limitations of our study because of small grafts ($10 \times 10 \text{ mm}^2$), short follow-up, and small sample size. Another limitation was the absent blinding of the investigators. However, the investigator bias is not apparent because the study reports negative results. Another reason to choose the same surgeons for the implantation and explantation of HAM was their expertise to find the difficult region of interest during the postmortem examination. Because an innovative proof-of-principle study is presented, a small number of cases were used. HAM regeneration in large constructs and anastomosis protection with improved sealing techniques in a high-risk situation (eg, peritonitis model) need further evaluation.

In conclusion, we suggest that HAMs alone are not a suitable barrier to prevent adhesions and inflammation and, therefore, are not beneficial to a standard suture for the reconstruction of the bowel wall in the presented xenograft model.

Acknowledgments

The authors highly appreciate the outstanding support throughout the experiments from Albert Ramon from International Tissue Engineering Research Association, Jin Yang from Affiliated Hospital of Chengdu University, China, and Peter Ponsaerts, Laboratory of Experimental Hematology, University of Antwerp, Belgium. Special thanks for the laboratory support go to Annette Wiggen-Kremer, Institute of Tissue Engineering and Regenerative Medicine (ITERM), Lukas Hospital Neuss, Germany.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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Original Research

Registry of implants for the reconstruction of pelvic floor in males and females: A feasibility case series

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H I G H L I G H T S

- A new web-based registry for the evaluation of implant assisted surgery for POP and SUI in males and females is presented.
- The presented case series show the feasibility of the registry with the need for indication based evaluation.
- The maximum score of cure was reached by 25–100% of patients depending on the indication.
- The preliminary results support the initiation of prospective registry according to IDEAL.

A R T I C L E I N F O

Article history:

Received 12 February 2017

Received in revised form

10 April 2017

Accepted 13 April 2017

Available online 15 April 2017

A B S T R A C T

Introduction: Most aspects of implant-assisted reconstruction of pelvic floor in males and females are under debate and the research is not standardized. Registries are supposed to shed light to the indications, surgical techniques and material properties and to establish a standardized evaluation.

Methods: A working group was formed to create an online platform for registration and outcome measurement of implant-assisted operations for pelvic organ prolapse (POP) and female and male stress urinary incontinence (SUI). 20 patients with modified mesh materials were evaluated over 23 months

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Keywords:

IDEAL
Implant
Incontinence
Mesh
Pelvic floor
Prolapse
Registry

follow up in the registry to prove the feasibility of the registry. For validation a previously published modified “satisfaction, anatomy, continence, safety – S.(A.)C.S score” was used.

Results: A consensus was met on definitions and classifications of patient variables, surgical procedures and implants, as well as outcome parameters (efficacy, continence, satisfaction, complications). Different subgroup modules were formed in accordance with treated condition. The maximum score of cure was reached by 25–100% of patients depending on the indication.

Conclusion: A prospective registry in accordance with IDEAL-D framework is justified for the evaluation and regulation of implants for pelvic floor reconstruction.

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Abbreviations

EDTA	Ethylenediaminetetraacetic acid
EuraHS	European Registry for Abdominal Wall Hernias
FDA	Food and Drug Administration
GCP	Good Clinical Practice
GeSRU	German Society of Residents in Urology
ICS	International Continence Society
IDEAL	Idea, Development, Exploration, Assessment, Long-term
IUGA	International Urogynecological Association
PGI-I	Patient Global Improvement Inventory
POP	Pelvic organ prolapse
POP-Q	Pelvic organ prolapse quantification
PROM	Patient Related Outcome Measures
QoL	Quality of life
RCT	Randomized Controlled Trial
S.A.C.S.	Satisfaction Anatomy Continence Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SUI	Stress urinary incontinence
TOT	Transobturator Tape
TVT	Tension-free Vaginal Tape

1. Introduction

To reduce the risk of recurrence, mesh-assisted repair of the pelvic floor has been introduced since the 1990's. First official approval of meshes by the Food and Drug Administration (FDA) dates back to 2003. To legalize the application of various prolapse and incontinence meshes the FDA approved a premarket equivalence notification 510(k). No clinical testing was demanded for the approval. In the last decade, the growing number of mesh operations and various presumed easy-to-use mesh kits from various manufacturers led to a widespread application of this outpatient surgical method [1,2]. Less attention was paid to possible new complications and only a few clinical trials were available prior to product approval and application [3,4].

Several FDA warnings from 2008 to 2016, reported on significant number of serious complications after the application of vaginal meshes or slings for POP and SUI repair. They proposed a higher risk-class for the approval of these medical products [2,5]. FDA reported mesh related complications including chronic pain, mesh infection, dyspareunia and long-term complications (mesh erosion and shrinkage), which were not analyzed in available studies. First, there was almost no reaction of the industry and surgeons to these warnings. Meanwhile, many manufacturers are confronted with a total of more than 100.000 law suits [6]. The consequence was a decrease of up to 40–60% implant-assisted operations mostly in the

USA and this trend spills over into Europe and other continents [7]. Moreover, FDA released another announcement in 2016, demanding clinical trials prior to application of vaginal meshes for prolapse surgery. Otherwise, the products would be abandoned from market approval in the USA [5]. The scientific societies reacted and proposed a cautious application for alloplastic materials. Standardized classification of mesh related complications was proposed by International Continence Society (ICS) and International Urogynecological Association (IUGA) [3]. European Commission assigned the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) to clarify the safety of surgical meshes in urogynecology. The current release notifies the insufficient scientific data and proposes a better education and the conduction of long-term trials, guidelines and registries (http://ec.europa.eu/health/scientific_committees/consultations/public_consultations/scenihr_consultation_27_en.htm) (last access 25.07.2016).

An outstanding example for the evaluation and regulation of surgical products and techniques is the IDEAL system of surgical innovation, which proposes an adequate Good Clinical Practice (GCP) – similar process of evaluation and approval of surgical techniques and medical devices. The method was initially described 2009 by Peter McCulloch and includes 5 consecutive steps of innovation: preclinical stage (Stage 0), idea (Stage 1), development and exploration (Stage 2), assessment (Stage 3) and long-term follow up (Stage 4) (Fig. 1) [1]. An IDEAL-D(evice) framework on the evaluation of medical devices has been published recently [8].

Herewith, we present the first application of IDEAL-D framework for the evaluation of urogynecological implants. A case series with an early registry is introduced to prove the feasibility of IDEAL-D system. The registry includes all implants for male and female incontinence and female prolapse surgery.

2. Materials and methods

2.1. Expert panel

Based on the successful implementation of surgical hernia registries, German quality assurance system and registry for hernia surgery (Herniamed) and European registry for abdominal wall hernias (EuraHS), a working group was formed to create an online platform for registration and outcome measurement of operations with application of implants for POP and SUI repair. Development of the registry involved reaching agreement on clear definitions and classifications of patient variables, surgical procedures and implant materials used, as well as outcome parameters, the triple P-triangle of pelvic floor reconstructions (Fig. 2) [9]. The working group comprised of an interdisciplinary expert panel under auspices of the German Society of Residents in Urology (GeSRU) and the Study Group for Urogynecology and Plastic Pelvic Floor

Fig. 1. IDEAL-D framework of the evaluation and regulation of medical devices [8].

Fig. 2. The triple P-triangle of pelvic floor repair, adopted from EuraHS [9].

Reconstruction (AGUB) of the German Society for Gynecology and Obstetrics. Over several working group meetings, consensus was reached on ICS and IUGA - based definitions and parameters for the data to be recorded in the registry [10]. Existing classifications were used where possible. However, many variables have been described, defined and classified by the working group.

2.2. Registry

The scope of the registry will include all surgeries for SUI and POP repair with application of implants: vaginal and abdominal meshes, female and male slings, artificial sphincter, reconstructive surgery with alloplastic materials. Adult male and female patients

older than 18 years should be included. The database will be used on a voluntary basis. A stratification of users will be offered. A Level 1 user will only have a small number of compulsory data fields to complete the registration of a case. These data will involve the variables needed for classification of incontinence and pelvic prolapse, the surgical technique and the materials used during the repair. Uploading a case should only take a few minutes. A Level 2 user will have the availability to complete a more comprehensive number of variables including detailed medical history, risk factors, detailed information on surgical technique and complications.

The surgeon uploading a case using his or her account will be the owner of the data. Various statistical analyses can be started from the web interface. The users will be able to extract their data in tables and in diagrams. Acknowledgement of the database as the source of the data has to be made every time it is used in public or in publications. However, the registry will not contain personal data like names or date of birth and will thus be completely anonymous. The link between the registration number and the patients' identity and the regular follow up 6 weeks, 6 months and annually after the initial surgery will be the responsibility of the user. To simplify the follow up, patients and referring physicians will be interviewed by telephone or mail. Additionally patients will be provided with implant identity cards. Pop-up windows with explanations of definitions, figures etc. are available on the platform. A registry logo is agreed upon and a website (<http://winc030.informatik.uni-wuerzburg.de:8080/>) with access to the database is provided (Fig. 3). The online platform for the registry was developed at the department of Artificial Intelligence and Applied Informatics, part of the Institute for Mathematics and Computer Science, at the University of Würzburg in Germany, under the supervision of Prof. Dr. Frank Puppe. The data were stored at the secured server area. A test-phase on the performance of the platform has been conducted by the working group members from October 2015 till March 2016. The work has been reported in line with the PROCESS criteria [11]. Additionally the study was registered at Research Registry, registration number researchregistry1149.

2.3. Ethics

All procedures performed in this study were in accordance with

Fig. 3. Logo of registry of urogynecological implants.

the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. An ethics committee vote is not necessary for this feasibility study, as a retrospective design was chosen to present the details of registry formation.

2.4. Patients

After obtaining corresponding informed consent, the first 20 patients (16 females and 4 males) with the application of a modified mesh-assisted SUI and POP repair have been evaluated as a consecutive case series and included in the registry. A retrospective study design was applied to evaluate the feasibility of the registry. Different mesh materials were used (TVT[®], Seratim[®], Ultrapro[®], Vitamesh[®]) and were all modified by a new technique with pre-operative surface coating with autologous plasma [12]. The technique has been evaluated previously in accordance with preclinical IDEAL stage 0 [13]. For the purpose of implant coating, 20–40 ml blood sample were obtained in the EDTA-tube (Ethylenediaminetetraacetic acid) from the respective patient by vein puncture before the induction of anesthesia and the clear supernatant (plasma) after centrifugation of the precipitation was removed with sterile syringe. Before the implantation the meshes were incubated for 30 min with 10–20 ml (depending on the size of the mesh) autologous plasma. The surgical technique was not altered by the application of this technology. The patients were examined pre- and postoperatively and interviewed before the operation and on telephone or per email after the operation.

2.5. Outcome evaluation

In reporting the goals of the surgery a previously published, practicable and timely S.A.C.S. score was adopted and used 24 months after the initial prolapse surgery, for the evaluation of male and female incontinence a modified S.C.S. (satisfaction–continence–safety) - score was used [14]:

– *Satisfaction*: to test patient's perception of success and the subjective measure of satisfaction, we used the validated version of Patient Global Improvement Inventory (PGI-I) scale. The surgery was defined as successful, if the patient felt “much better” or “very much better” after the surgery. Value = 1.

– *Anatomy*: to verify anatomical success of POP surgery, we used the POP–Q, which refers to an objective, site-specific system for describing, quantifying, and staging pelvic support in women. According to the POP–Q, we used very stringent criteria for the definition of complete success (the absence of any ≥ 2 stage prolapse). This parameter was only used for female POP evaluation. Value = 1.

– *Continence*: for the definition and grading of postoperative continence, we administered the validated ICIQ-SF2004 questionnaire, post-operative pad use and the Ingelman-Sundberg scale (score 0–3); for the score, we considered a complete success the absence of any degree of urine leakage and the use of no pad or only a protective pad. Value = 1.

– *Safety*: to analyze the safety of the procedure, we used the ICS/IUGA and Clavien–Dindo-classification of surgical complications (score 0–5) [7,15]. The absence of perioperative or delayed revision surgery due to a complication except for intraoperative bladder perforation (commonly not assumed as a severe complication) was defined as success. Value = 1.

Each component of the scoring system produced a binary nominal categorical variable (1 or 0). Perfect scoring systems, according to the S.(A.)C.S., were defined as the sum of satisfaction (plus anatomy) plus continence plus safety, with a total score of 4 for POP and a total score of 3 for SUI representing a ‘cure’. The degree of concordance among these scores was estimated using Cohen's kappa test of inter-rater agreement (with k-values ranging from 0 to 1).

3. Results

All pelvic floor reconstruction surgeries with application of implants like abdominal and transvaginal mesh or biological materials, male and female sling, and artificial sphincter were included and different modules were created. A set of well-described definitions, risk factors for recurrences and complications was listed. A comprehensive information on management strategies for complications can be provided by the user in Level 2. An online platform with statistical analysis was established, which can be used by individual surgeons, teams or for multicentre studies. The questionnaires were evaluated for the perioperative data of the first 20 patients. The first results showed the feasibility and the timely application of the registry. The data was entered by 3 different users, all Level 2 with minimum 2 follow ups after the initial implantation. All follow ups were done by telephone interview, a form of patient related outcome (PROM). The data on postoperative anatomy outcome was retrieved from referring physicians. The mean time for the data input (Level 2, extended) in the database was 18 min (range 12–26).

Between 04/2013 and 05/2014, 20 patients (16 females and 4 males) with the indication for SUI and POP repair with mesh graft were selected for surgery in a single institution. The mean age was 67 years (45–85) and the mean follow up was 23 months (17–29). 11 patients were treated for SUI (grades II–III, Stamey score) and 9 patients were treated for POP (POP–Q grades II–III, anterior and apical prolapse).

Three reoperations (15%) were needed due to complications (2 postoperative obstructions after TVT-procedure, 1 abdominal hernia 12 months postoperative after abdominal sacrocolpopexy). No other severe complications (mesh exposure, bladder or bowel injury, and fistula) were registered. Two reoperations (10%) were needed for persisting incontinence or prolapse (Table 1).

According to the S.(A.)C.S. scoring system, only 13 patients (65%, Table 1) reached the cumulative perfect score of 3 for SUI or 4 for POP at 24-month follow-up: high PGI-I score, no residual prolapse ≥ 2 according to the POP–Q staging system, no pad use (or no more than one protective pad), and no grade >2 complication nor delayed surgery related complications. However, significant differences between the procedures could be found with maximum 100% for anterior vaginal mesh vs. 25% for TOT male incontinence surgery. The highest agreement with S.(A.)C.S. score was reached for the satisfaction component of the score (Cohen's $\kappa = 0.88$).

Table 1
Classification of complications and S.(A.)C.S. score [14].

Procedure	TVT	TOT	Ant. Vag. mesh	Sacropexy	Total	IUGA/ICS-class.
Number of patients (gender)	7 (female)	4 (male)	1 (female)	8 (female)	20	
Complications, number (%)						
Clavien-Dindo Grade I						
Prolonged pain	0	1 (25%)	0	1 (12.5%)	2 (10%)	6Bd T2 S3/S4
Hematoma	1 (14%)	1 (25%)	0	0	2 (10%)	7A T2 S2-S4
Urge de Novo	3 (43%)	1 (25%)	0	0	3 (15%)	4B T2
Obstructive micturition (prolonged catheter)	1 (14%)	0	1 (100%)	0	2 (10%)	4B T2
UTI	2 (28%)	0	0	2 (25%)	4 (25%)	4B T2
Clavien-Dindo Grade II Wound infection	0	0	0	1 (12.5%)	1 (5%)	6Cb T2 S4
Clavien-Dindo Grade Grade III						
Obstructive micturition	2 (28%)	0	0	0	2 (10%)	4B T2
Hernia	0	0	0	1 (12.5%)	1 (5%)	6Bd T3 S5
Reoperation for SUI/POP	0	1 (25%)	0	1 (12.5%)	1	
Clavien-Dindo Grade Grade IV-V	0	0	0	0	0	
S.(A.)C.S score						
Satisfaction	6 (86%)	1 (25%)	1 (100%)	6 (75%)		
Anatomy	na	na	1 (100%)	6 (75%)		
Continence	7 (100%)	1 (25%)	1 (100%)	8 (100%)		
Safety	5 (71%)	4 (100%)	1 (100%)	7 (87%)		
S.(A.)C.S score 4	5 (71%)	1 (25%)	1 (100%)	6 (75%)	13 (65%)	
S.(A.)C.S score 1–2	2 (14%)	3 (75%)	0	2 (25%)	7 (35%)	
na, not applicable						

4. Discussion

A restart for the application and indication of alloplastic materials for pelvic floor reconstruction and standardized quality trials are needed urgently. We need to know the quality of our surgeries and educate the patients properly about possible complications. Mesh-related complications like erosion, exposure, infection, pelvic pain and mesh shrinkage should be considered and risk factors should be identified [3]. However, there is a number of late mesh-related complications, so that a long-term follow up is important for a proper evaluation of the procedure [16].

There are several reasons for the preliminary fail of the implants in urogynecology. Different modifications of a surgical technique, indications and follow up are not standardized. The main problem is the approval of medical products. The pharmacological studies are regulated by strict rules requiring phase I-III studies according to GCP. The medical products are less strictly regulated and can get approval without quality studies [1,6]. However, the difficulty is to evaluate medical devices with a clinical study. It is possible to look for a correlation between the medicament and a symptom in a pharmaceutical trial. In contrast, the result of a surgical procedure depends on many different factors. Thus, numerous subgroup and multivariate analyses with large patient cohorts would be necessary. Randomized clinical trials (RCT) remain the source of the best evidence for pharmaceuticals. In contrast, they are not always practicable for medical products. In a RCT, the randomized controlled variable is just one out of many. The long delay from surgery to the development of many complications such as recurrence and the impossibility to control all relevant parameters can hinder proof of the significant impact, in particular, when studying slight modifications of techniques or materials.

A functional solution with a simple approval of medical products without restrictions concerning the products efficacy and the safety of patients is obviously required. IDEAL-D is a simple and practicable system for the evaluation of medical devices. Surveillance by registries from small series would be an important part of IDEAL and would allow opportunities for using risk adjustment techniques to analyze large registry datasets to study small or long term effects in situations with multiple confounders, in which a randomized trial might be infeasible [8]. However, projects like IDEAL are still at their beginning and consensus on key outcomes (e.g.

functional results, scope and severity of complications) as well as contextual factors (e.g. grading of patient risk factors, severity of comorbid pathology or general health, details of surgical technique and perioperative setting) will need consensus among specialist communities and specialties as well as journals in order to standardize reporting accordingly.

A registry allows the detection of poor and good results, if they appear more frequently than expected. Surgical hernia registries in general and national registries, like the Swedish TVT database and the Austrian TVT database have been previously successfully established and improved the quality of surgery after the implementation [17,18]. However, the Austrian registry was timely limited and Swedish registry is obligatory and under auspices of the government. The Austrian and Swedish models are not transferable to other bigger countries. Hereby, we confirm the feasibility of the presented registry with the possibility of timely and effective data entry. Further, we adopted a previously published simple score system to evaluate the surgical technique for POP and SUI repair. The 4-point S (satisfaction), A (anatomy), C (continence), S (safety) - score was presented by Mearini et al. to evaluate 233 women 24 months after open sacrocolpopexy [13]. The authors detected the sensitivity of 74.1%, the specificity of 90% and a total diagnostic capacity of 75.5% for the new score. According to the S.A.C.S. scoring system, only 160 patients (68.6%) reached the maximum score of cure in the above mentioned study. We applied the score for female POP implant surgery and modified it for female and male SUI implant surgery and used here a simplified S.C.S. score for evaluation, as the anatomy cannot be considered. In our collective, 13 patients (65%, Table 1) reached the cumulative perfect score of 3 or 4 at 24-month follow up, these results are similar to the data of Mearini et al. and were stable after 24 months. We found as well a strong agreement of the score and satisfaction. However, an internal validation was not applicable due to a heterogenic and small patient collective.

The main limitation of this study is the inclusion of male and female with different POP and SUI methods, which should be evaluated on each own. Further statistical analyses like logistic regression to analyze the possible risk factors for the outcome were not applicable due to small patient numbers. Another problem is the future interpretation of the results. Less than 70% of the whole group reached a perfect S.(A.)C.S. score, in the male SUI group only

Fig. 4. Registry expansion process.

25%. Predetermined lowest score rates (cut-off) are necessary for the future to decide which procedure or implant does not meet the quality standards. Another limitation is the large time amount required for the data input. A timely solution with effective questionnaire are needed for high and consistent study number. However, first results show the feasibility and the value of the database. The aim of our study was to present a preliminary model on how to establish a long-term database for the evaluation of new implant techniques for POP and SUI repair in female and male. However, further proofs and internal and external validations on large collectives are necessary. A prospective registry-based evaluation of different indications and procedures, as described in this study, will shed light into the role of implants for the reconstruction of pelvic floor. The evaluation of the registry should be done by an independent committee under auspices of national and international scientific societies. The funding, obliged to register application of implants and regulation of the approval process should be provided by Medical Device Regulation authorities.

Given the fact, that IDEAL is an international collaboration, a fast international spread and improvement of the registry by publications and congress presentations can be expected. Recently a first outcome of the registry with a randomized trial protocol, exploring a new mesh improvement for pelvic organ prolapse surgery, has been published [19]. The participating scientific societies and industrial manufacturers will expand and update the registry towards a multi-country or multi-continent presence (Fig. 4). Our group is a member of “the national dialogue on implant register”, which is a part of a national strategy process “Innovations in medical technology” supported by the Federal Institute for Drugs and Medical Devices, the Federal Ministry of Economics and Technology, the Federal Ministry of Education and Research and the Federal Ministry of Health. The meshes will be classified as a class III high-risk product by the upcoming EU medical device regulation and the postmarket follow up data will be required prior to approval. An implementation of quality depending reimbursement is planned by the politics. A successful registry based on the experience and consent of participating gynecological and urological societies will pave the way for the mandatory registry system driven by politics and with the financial support of the

participating industrial manufacturers.

Registries according to the IDEAL method of surgical innovation allow a standardized follow up of different techniques and implants and a better preoperative counseling of patients by surgeons, based on each individual's clinical situation.

Ethical approval

Not needed due to retrospective design.

Funding

No funding.

Author contribution

Dimitri Barski and Holger Gerullis: Project development, Data Collection, Manuscript writing, Register harmonising to IDEAL criteria, Statistical analyses.

Thorsten Ecke and Ralf Joukhadar: Project development, Manuscript editing, Register harmonising to ICS/IUGA guidelines.

Jennifer Kranz, Rana Tahbaz, Fabian Queissert, Laila Schneidewind, Sandra Mühlstädt, Markus Grabbert, Nadine Leistner, Alexander Pelzer, Uwe Klinge: Project development, Manuscript editing.

Laila Schneidewind: Statistical analyses.

Werner Bader and Gert Naumann: Project development, Manuscript editing, Register harmonising to ICS/IUGA guidelines.

Frank Puppe: Development of the website and online support

Mihaly Boros and Thomas Otto: Project development, Manuscript editing.

Conflicts of interest

No conflicts.

Guarantor

Dimitri Barski and Holger Gerullis.

Research registration unique identifying number (UIN)

Researchregistry1149.

Acknowledgements

All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this manuscript, take responsibility for the integrity of the work as a whole, and have given final approval to the version to be published. This work is a part of a national strategy process "Innovations in medical technology" supported by the Federal Institute for Drugs and Medical Devices, the Federal Ministry of Economics and Technology, the Federal Ministry of Education and Research and the Federal Ministry of Health. Special thanks goes to the extended collaboration group of "the national dialogue on implant register". Dimitri Barski and Holger Gerullis contributed equally to this manuscript.

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