Urogenital tissue engineering using new hybrid biomaterials

door

Paul Kees Jan Dik de Jonge

The work presented in this thesis was carried out within the Radboud Institute for Molecular Life Sciences at the department of urology, Radboud University Medical Center, Nijmegen under the supervision of Prof. dr. W.F.J. Feitz, dr. E. Oosterwijk and dr. P.J. Geutjes. The research presented in this thesis was financially supported by the Radboudumc PI program.

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Contents

Chapter 1

General introduction and thesis outline

General introduction

Urogenital system, pathologies and current treatment options

The urogenital system is composed of the reproductive organs and the urinary system. These organ systems are often grouped together due to their common embryological origin, their proximity and the use of organs like the male urethra for both organ systems [1]. This thesis focuses on three organs from this system: The ureter, the urinary bladder and the urethra (figure 1). Like most organs, these are susceptible to acquired or congenital pathologies.

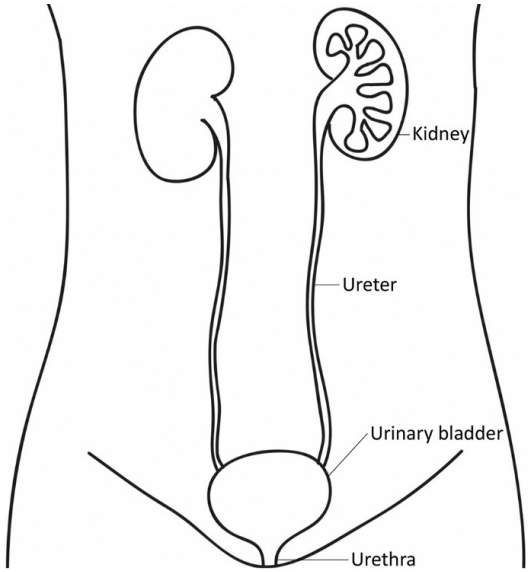

Figure 1. Schematic overview of the urinary tract. Urine is produced by the kidneys and is transported through the ureters to the urinary bladder. The urinary bladder collects the urine, which is voided through the urethra when full.

The ureter

The ureter is a muscular hollow tube that actively transports urine from the kidney to the urinary bladder through peristalsis [2]. Valves at the entrance to the bladder prevent reflux of urine. Ureteral damage mainly results from acquired pathologies such as injury, tumors or iatrogenic injury during surgical procedures [3]. A large retrospective analysis between 2002 and 2006 showed that 2,6% of all urogenital traumas involved the ureter [4]. While ureteral injury is relatively uncommon, failing to recognize the injury can lead to severe side effects, including sepsis or loss of renal function [5]. Specific anatomic characteristics of the ureter, such as the segmental vascular supply and the lack of native tissue, limits surgical ureteral reconstruction. Several surgical techniques have been introduced to repair long ureteral defects. Common procedures include a ureteroneocystostomy, a Boari flap, ileal interposition and renal autotransplantation. When these procedures fail, an undesirable nephrectomy often is the only option [6, 7].

The urinary bladder

The urinary bladder is a hollow muscular organ located in the pelvic floor. It collects and stores up to 300-500ml urine produced by the kidneys [2]. Acquired pathologies of the urinary bladder often result from tumors and can require complete resection of the urinary bladder [8]. Congenital anomalies can result from diseases such as bladder exstrophy or a neuropathic bladder [9]. Patients with congenital anomalies often require bladder augmentation to increase the volume of the bladder. Augmentation is most often performed using gastrointestinal tissue. The use of this tissue can be problematic due to a lack of tissue or result in side effects such as metabolic abnormalities, infections or stone formation [10, 11].

The urethra

The urethra is a hollow muscular tube that is used to void urine from the urinary bladder [2]. Congenital defects of the urethra, such as hypospadias, and acquired defects, such as strictures are major clinical problems. Hypospadias occur in 1 in 300 live male births and 1 in 1000 men > 65 years of age develop strictures [12-14]. Current treatment usually involves surgery and often requires graft tissues such as local tissue flaps or buccal mucosa [15-17]. The use of autologous grafts is often associated with donor site morbidity and complications such as infections or recurrent strictures [18].

Tissue engineering

While treatment options are available for most acquired and congenital pathologies of the urogenital system, there is a clear need for new treatment options to reduce side effects and recurrences. Tissue engineering and regenerative medicine aims at repairing or replacing damaged organs or tissues [19]. These newly formed tissues should provide similar functionality as the native organ or tissue. In general tissue engineered material can be produced by decellularization of donor tissue or by de-novo preparation from natural or synthetic materials [20-22]. Currently, no superior material has been identified. Several attempts have been made to improve these materials. These include the culture of stem) cells in the materials or the addition of biologically active materials such as growth factors and glycosaminoglycans (figure 2) [23, 24].

The ureter

While the literature on urogenital tissue engineering is quite extensive, the ureter is relatively understudied. This may suggest that the ureter is a challenging tissue to reconstruct due to the complexity of creating an actively contractile and tubular tissue [7]. Another reason may be the relatively low number of patients that need a tissue engineered ureter. In chapter 2 of this thesis it was demonstrated that the evidence in the literature was inconclusive about the optimal tissue engineering approach to treat long ureteral injuries. Clearly, there is still a need to develop new tubular constructs for ureteral repair.

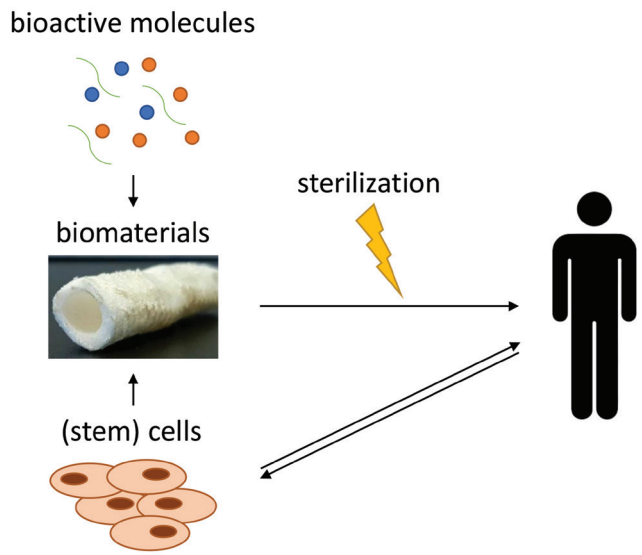

Figure 2. Schematic overview of tissue engineering approaches. In general, biomaterials are used as a scaffold or template to support the function of the organ while the tissue is regenerating. These materials can be enhanced with (stem) cells or bioactive molecules which may improve tissue regeneration. Before a material can be implanted into a patient, sterilization is required.

The urinary bladder

Contrary to the ureter, tissue engineering of the urinary bladder has been studied more extensively. Atala *et al.* performed the first clinical trial using tissue engineering to improve bladder capacity [25]. While these results were promising, they could not be replicated in a phase 2 study [26]. Another clinical study showed that it was possible to improve bladder capacity, but the primary clinical endpoint of improved dry intervals was not reached [27]. This shows that improvements to the design of the tissue engineered constructs are still required before clinical trials can be considered again [28]. A systematic review of pre-clinical studies showed animal models with dysfunctional bladders in large animal models are required to properly test new tissue engineered constructs [29].

The urethra

The urethra is probably the most studied organ of the urogenital tract regarding tissue engineering. More than 20 clinical studies have been published using different materials and techniques as shown in chapter 4 of this thesis. Despite promising results, tissue engineering is still not routinely applied in clinical practice. This may be explained by insufficient quality of reporting in pre-clinical studies and a lack of proper control groups. To improve clinical translation, the quality of pre-clinical studies and their design should be improved.

Design of tissue engineered constructs

Scaffold design has a major impact on the *in vivo* performance of tissue engineered constructs. Obviously, the shape, size and mechanical requirements depend on the organ that is being treated. The materials, cells and other factors used show great similarities between urogenital tissue engineering applications as these organs share many features e.g. they are hollow, their function depends on smooth muscle tissue and urothelium protects the surroundings (figure 3).

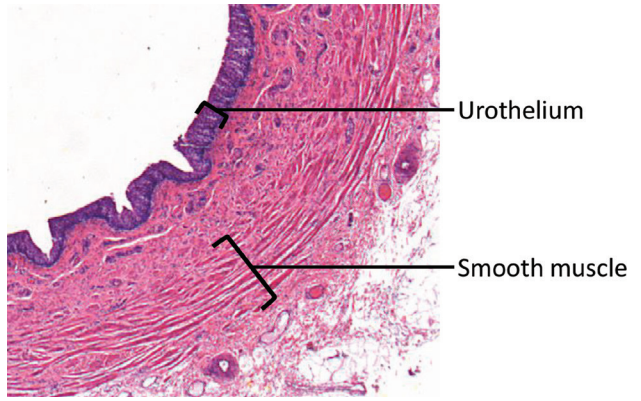

Figure 3. A histological cross section of the ureter showing the two most important functional tissues in the urogenital organs. The smooth muscle tissue is responsible for active transport of urine through contraction. The urothelium forms a barrier between the toxic urine and the surrounding tissues. Scalebar = 400µm.

Materials

The first consideration when designing a tissue engineered construct is which material to use. The material is the major component of the constructs and acts as a permanent or temporary scaffold during the regeneration process of the body [30]. Ideally, the materials should be biocompatible, biodegradable and stimulate tissue regeneration. Materials should have similar biomechanical properties to the tissue that is being repaired to prevent fibrosis or extrusion of the material [31]. Many different materials have been studied, either alone or in combination, to achieve the desired properties. Common materials for soft tissues include decellularized tissues such as small intestinal submucosa and acellular bladder matrix, natural materials such as collagen or synthetic materials like polyglycolic acid and poly(lactic-co-glycolic acid) [32-36].

Every type of material has its own advantages and disadvantages. Decellularized tissues generally have an intact native architecture and may present naturally occurring bioactive molecules to improve regeneration [37], but molding in the desired dimensions may be difficult. In contrast, constructs produced in the laboratory from natural materials like collagen are highly tunable and material properties can be adjusted towards the application at hand. Finally, synthetic materials offer most control over material properties such as degradation speed and mechanical strength [38]. However, these materials are foreign to the body and may induce a more severe immune response than natural materials.

This thesis mainly focused on the use of collagen and collagen combined with poly(lactic-co-glycolic acid) to control the mechanical and degradation properties.

Implantation techniques

In urological tissues, the urothelial cells and smooth muscle cells are specifically important for the function of the organs [39]. When designing tissue engineered constructs, it is possible to culture cells on the biomaterial before implantation. Initially, primary cells were mainly isolated from autologous tissue. These cells however, are not always sufficiently available and may be compromised in case of malignant disease. Therefore, it was recently shown that mesenchymal stem cells derived from e.g. bone marrow or adipose tissue are a promising cell source as they are abundantly available and can differentiate to multiple cell types [40-43]. Pre-conditioning of constructs with cells may be used to improve the regeneration outcome [44, 45].

Currently, tissue constructs without cells are mostly directly implanted to repair the damaged organ or tissue as an off-the-shelve solution. While this is generally possible for small defects, it may become problematic when large constructs are needed because vascularization is absent [46]. Without vascularization of the tissue, oxygen and nutrients can only reach the tissue construct by diffusion, risking necrosis and central fibrosis [47]. Several methods have been described to improve the vascularization of tissue engineered constructs, including the addition of growth factors that play a key role in the formation and maturation of blood vessels [48]. Other options include pre-implantation of the construct whereby the host body is used to generate a vascularized graft. In this approach, the engineered construct is implanted in a non-functional location such as the omentum, peritoneum or subcutis. After a few weeks, the tissue is then transplanted to the functional location. By using this technique, partial remodeling including blood vessel formation and the initial immune response can occur without negatively impacting the function of the tissue [25, 49-52]. While the use of cells and pre-implantation techniques has shown promising results, it may not always be applicable due to the incubation times. In these cases, off-the-shelve solutions are still required.

Animal models and clinical translation

The use of animal models is a critical step in the evaluation of the safety and performance of tissue engineered constructs as the complexity of a body during remodeling cannot be simulated *in vitro* yet [53]. Choosing the right animal model and using proper experimental design are paramount to successful clinical translation as shown in chapter 4 of this thesis. Moreover, for tissue engineered constructs to be implemented in a clinical setting it needs to meet all regulatory requirements related to medical devices [54]. This includes sterilization for which multiple techniques are available. Common techniques include autoclaving, gamma irradiation and ethylene oxide gas. Not all techniques are compatible with materials. Autoclaving at high temperatures may degrade the biomaterials [55]; gamma irradiation can create or break cross-links in the material, adjusting the intrinsic material properties and ethylene oxide can leave residues that are toxic [56-68]. It is therefore critical to take the clinical regulations and demands into account in the design of tissue engineered constructs and experiments.

Thesis objective and outline

The aim of this thesis was to develop new technologies and medical devices to treat patients with severe urogenital trauma as an alternative to current graft tissues in reconstructive surgery.

In this thesis, new urogenital tissue engineering and regenerative medicine applications are described. In the first part the current state-of-art for ureteral and urethral tissue engineering through (systematic) literature searches is discussed. In the second part, the use of newly developed tubular and flat collagen based (hybrid) constructs *in vivo* and *in vitro* is presented.

Part 1: A comprehensive overview of urogenital tissue engineering

The first part of this thesis was focused on identifying the current state-of-art in urogenital tissue engineering.

In **chapter 2** a literature review on ureteral tissue engineering was performed. In this review, a trend was identified showing a shift from non-degradable synthetic materials to degradable natural biomaterials such as collagen and decellularized tissues.

Chapter 3 focused on new developments in ureteral tissue engineering in the last 3 years. Where mainly simple constructs without cells were previously used, most new studies focused on the use of stem cells seeded on decellularized tissues. In addition, the constructs were often implanted in a non-functional location such as the subcutis or the omentum for preconditioning. While these techniques showed promising results, there were only few studies published in the field of ureteral tissue engineering.

Chapter 4 systematically reviews the most studied field of urogenital tissue engineering for pre-clinical and clinical studies: the urethra. In this study the relation between preclinical and clinical studies was investigated, identifying current strengths and weaknesses in study design and execution.

Part 2: New hybrid biomaterials in urogenital tissue engineering

After having identified the current state-of-art in urogenital tissue engineering, new collagen based medical devices were developed and evaluated *in vitro* and *in vivo*. First, relatively simple flat constructs were evaluated.

In **chapter 5** porcine smooth muscle and urothelial cells were cultured on flat collagen constructs comparing traditional static cell culture and dynamic cell culture in a bioreactor. By mimicking normal tissue conditions, proliferation and differentiation of the cells could be influenced.

In **chapter 6** the performance of flat collagen patches to increase the total bladder capacity was evaluated in a pig model. Multiple constructs were used to increase the total surface area of the bladder. Three conditions were studied: constructs containing collagen only, collagen with heparin and collagen with heparin and growth factors.

Next, more complex tubular constructs were developed and evaluated *in vivo*. **Chapter 7** describes the outcome of the repair of a created critical five-centimeter-long ureteral defect in pigs using tubular collagen-Vicryl constructs. Half of the constructs were loaded with growth factors FGF-2 and VEGF to improve vascularization and smooth muscle cell ingrowth. Unfortunately, the outcome was sub-optimal. Because of the size and weight of pigs, young animals had to be used. These animals grew rapidly during the experiments, which may have influenced the regeneration process.

Therefore, a similar approach was tested in an adult goat model. In **chapter 8** the first experiences with the goat model are described. Technical complications were encountered due to anatomical differences. By employing clinical protocols for stent placement, these complications could be avoided.

Chapter 9 describes the results of ureteral reconstruction in goats. In light of recent advancements in the field of tissue engineering, collagen-Vicryl constructs that were directly implanted in the ureter were compared to constructs that were first implanted in the subcutis for one month. This pre-implantation helps to partially replace the construct with native tissue and increases vascularization.

Finally, the results and conclusions are summarized in **chapter 10.** In addition, future perspectives for the development of tissue engineered constructs for urogenital applications are described.

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Part 1

A comprehensive overview of urogenital tissue engineering

Chapter 2

Ureteral tissue engineering: Where are we and how to proceed?

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Abstract

In the field of regenerative medicine, various types of biodegradable and nonbiodegradable scaffolds have been developed for urinary tract tissue-engineering applications. Naturally derived or synthetic materials have been tested to determine their properties and their effectiveness. However, the majority of the current literature focuses on the reconstruction of the urethra, urinary diversion, and urinary bladder, while limited data have been published regarding the use of biomaterials in ureteral reconstruction. Tissue engineering might offer alternative and less invasive therapeutic options for long ureteral defects compared with the current surgical reconstructive techniques and their potential complications. In this article, we aimed to review the literature regarding ureteral tissue engineering through a Medline search and describe new potential options for future clinical applications. We concluded that the available literature is inconclusive since the superiority of a specific scaffold has not been demonstrated and the latest developments of regenerative medicine have not been evaluated in ureteral tissue engineering yet.

Introduction

Damages to the ureters are caused either by an injury or a pathologic situation that may result in stricture formation, such as urolithiasis or chronic inflammation. Most ureteral injuries are iatrogenic due to surgical procedures or radiation therapy [1]. If identified, many of these lesions can be repaired primarily, but if left unrecognized, they can lead to sepsis or loss of renal function [2]. About 73% of all ureteral injuries occur during gynecological operations, most often during hysterectomy [3-7]. What is of great importance is that these complications often remain unrecognized (33% to 87.5% of cases) at the time of the surgery [8-10]. Oncological, vascular, and general surgery procedures can also result in intraoperative ureteral injuries [11]. Intra- and postoperative complications of endourological procedures have also been increased due to the increased number of diagnostic or therapeutic ureteroscopies performed worldwide during the last years. Ureteroscopy results in ureteral avulsion in 0.3% and perforation in 2% to 6% of cases. The perforation rates are lowest in series using smaller caliber ureteroscopies [12-15]. Long-term data regarding stricture formation due to endourological procedures are lacking. Injuries to the ureter by an external cause such as gunshot wounds, stab wounds, or blunt injuries are relatively uncommon (less than 1%) and in the majority of cases are combined with other organ lesions [15]. Penetrating traumas result in ureteral injury twice as often as blunt traumas. The mortality rates for penetrating and blunt ureteral traumas were 6% and 9%, respectively [16].

The site and the length of the affected ureter are of great importance for the surgical repair [17]. The classical surgical techniques for long ureteral defects (Boari flap, Psoas hitch, transureteroureterostomy, reimplantation, Blandy cystoplasty, and ileal interposition) are not always applicable and they also carry their own risks for complications such as recurrent strictures, urinary leakage, metabolic complications, and donor tissue harvesting problems. Since traditional surgical procedures for ureteral repair have their own limitations and complication rates, new therapeutic approaches are needed in ureteral surgery. Tissue engineering may contribute to ureteral reconstruction by developing new suitable tubular biomaterials that could serve as a ureter, and thus preserve the normal renal function.

In this article, we review the literature regarding ureteral tissue-engineering applications. A Medline search was performed for articles published between 1983 and February 2013 regarding ureteral tissue-engineering applications. Combined MeSH terms were ureter, biomaterials, tissue scaffolds, tissue engineering, regenerative medicine, and growth factors. Articles that examine the etiology and assessment of ureteral lesions were also included in the article. Finally, we propose new potential options for future clinical applications.

Ureteral Tissue Engineering Overview

The underlying supposition of tissue engineering is that the employment of the natural biology of the system will allow for greater success in developing therapeutic strategies aimed at the replacement, repair, maintenance, and/

or enhancement of the tissue function [17]. The biomaterials involved must maintain the physiological anatomy and functionality of the original tissue and have the proper mechanical and structural properties. They should also provide a microenvironment capable of supporting certain cell types. Under these conditions, cells will be able to differentiate and regenerate tissues according to their tissue of origin. Alternatively, smooth muscle cells (SMCs) and urothelial cells (UCs) may be seeded onto scaffolds to enhance tissue regeneration. In time, cells produce new extracellular molecules, which gradually replace the initial structured scaffold leading to a healing and regenerative process. Autologous cells are preferred due to biocompatibility, and thus, avoidance of tissue rejection.

In the field of regenerative medicine, ureteral tissue engineering remains an underreported topic so far. This could be due to either the smaller percentage of ureteral lesions or cross-over knowledge from the urinary bladder and urethral tissue-engineering studies. In Table 1, we present the ureteral tissue-engineering studies with regard to the type of construct used, animal model, scaffold length, regeneration outcome, and tubular or onlay application of the biomaterial.

Authors	*Animal model*	*Scaffold*	*Seeded*	*Length*	*Regeneration outcome*	*Technique*
Tachibana [1985][18]	Dog	Collagen	No	5cm	UC/F	T
Dahms [1997][19]	Rat	Collagen	No	0.3-0.8cm	UC/SMC	T
Baltaci [1998][20]	Dog	Gore-Tex	No	5-8cm	none	T
Sabanegh [1996][21]	Dog	Gore-Tex	No	10cm	UC (minimal in growth)	T
Osman [2004][22]	Dog	AM	No	3cm	UC/SMC	T
Liatsikos [2001][23]	Pig	SIS	No	7cm	UC/SMC	O
Smith [2002][24]	Pig	SIS	No	2cm	UC/SMC/F	O
Shalhav [1999][25]	Mini pig	AM, SIS	No	1.5-2.8cm	UC/SMC/F	T*
Duchene [2004][26]	Pig	SIS	No	2cm	UC/F	T*
Sofer [2002][27]	Pig	SIS	No	2cm	UC/SMC/F	T*
El-Assmy [2004][28]	Dog	SIS	No	4cm	UC/SMC/F	T*
El-Hakim [2005][29]	Pig, Dog	SIS AM DSB	Yes	3-5cm	F UC/SMC/F UC/BM	T*

Table 1. Preclinical studies regarding ureteral tissue engineering applications. AM= Acellular Matrix, DSB= Decellularized Small Bowel, T= Tubular, T*=Tubularized, O= Onlay, UC=Urothelial Cells, SMC=Smooth Muscle Cells, F=Fibrosis, BM=Bowel Mucosa

One of the first reported attempts to replace a ureteral segment with biomaterials was performed by Tachibana *et al.* They concluded that approximately 5-cm-long tubular collagen sponges in the canine ureter could promote the regeneration of UC layers with coeval SMCs regeneration present only at the junctional area between the primary ureter and the graft. No severe hydronephrosis was observed in six dogs where a ureteral stent was used, while in the remaining two dogs where no stent was used, severe strictures of the anastomotic sites were observed [18]. Another study by Dahms *et al.* examined the use of acellular collagen tubular scaffolds in a rat model. SMCs and nerve fibers were noticed at 10 and 12 weeks, respectively, while at 3 months, SMCs had assumed a regular configuration in a lower density compared to the normal contralateral ureter. The SMCs were arranged in parallel rows in the longitudinal direction with a decrease from the end to the central part of the scaffold. The ureteral segment that was replaced ranged from 0.3 to 0.8 cm. An examination of the specimens showed various degrees of hydronephrosis and this might be attributed to the migration of the stent to the distal ureter in all animals [19].

Baltaci *et al.* tested a 5- to 8-cm Gore-Tex ureteral graft in a canine model, but advanced hydronephrosis, atrophy of the renal parenchyma with calcium deposits, and no cellular growth was noted in all five animals. Although the lumen of the proximal and distal ureter was not obstructed, there was severe fibrosis and strictures at the proximal and distal anastomotic sites. The ureteral mucosa proximal to the Gore-Tex tubular graft revealed squamous metaplasia [20]. Gore-Tex was also evaluated in a canine model by Sabanegh *et al.* They used a 10-cm tubular graft and reported the absence of hydronephrosis in five of the total eight animals at 6 months or 1 year. Histology revealed a marked acute and chronic inflammatory reaction surrounding the graft, but the luminal diameter remained unaffected. Also, minimal cellular migration was noticed through the scaffold to the lumen [21].

Osman *et al.* tested a 3-cm tubular acellular matrix in a canine model. The constructs were prepared from heterologous canine ureters after cell lysis. Hydronephrosis, shrinkage of the graft and strictures with narrowing of the graft lumen were observed [22]. Small intestinal submucosa (SIS) has also been used for ureteral reconstruction. Liatsikos *et al.* replaced two-thirds of the diameter of a 7-cm ureteral segment in a pig model with SIS. They demonstrated epithelial regeneration supported by a prominent submucosal neovascularization. SMCs did not exhibit the normal organization found in the original ureter [23]. Smith *et al.* replaced half the diameter of a 2-cm-long ureteral segment in a pig model with SIS as an onlay patch laparoscopically. After 9 weeks, a primarily transitional epithelium was observed at the SIS graft with focal intestinal metaplasia. The submucosa and ureteral musculature appeared histologically normal [24]. Shalhav *et al.* also laparoscopically replaced a 1.5–2.8-cm ureteral segment with either the acellular matrix (prepared from mini pigs or domestic pigs) or tubularized SIS. They reported regeneration of urothelium, but also bone metaplasia with dense fibrosis and obstruction of the neo-ureter in all animals [25]. These findings were confirmed by Duchene *et al.* in a pig study, where a 2-cm ureteral segment was replaced laparoscopically by SIS. In contrast to tubularized SIS, where all animals demonstrated hydro-ureteronephrosis or renal atrophia, partial replacement of the ureteral wall with an SIS patch as onlay led to re-epithelization and normal appearance of the kidney [26]. Sofer *et al.* tested a 2-cm SIS graft tubularized over a 10F ureteral stent in a pig model. The histological evaluation demonstrated regeneration of both the urothelial and smooth muscle layers over the graft. However, this regeneration was associated with an intense fibrotic and inflammatory process resulting in complete ureteral obstruction and secondary hydronephrosis at 12 weeks postoperatively. In addition, mucous metaplasia of the epithelium, metaplastic bone and dystrophic calcification

of sloughed luminal fragments and mucosal ulceration were observed [27]. El-Assmy *et al.* replaced a 4-cm-long ureteral segment with a tubularized one-layer SIS graft in mongrel dogs. Regeneration of urothelial and smooth muscular layers was noticed with associated intense fibrosis and inflammation resulting in hydroureteronephrosis [28]. El-Hakim *et al.* published three sets of experiments. In the first set, they compared a 5-cm-long nonseeded versus seeded with autologous urothelial and SMC tubularized SIS in pigs. In the second set, they compared a 3-cm decellularized porcine ureteral segment seeded with autologous bladder cells versus nonseeded decellularized porcine ureteral segment in beagles. In the third set, they examined a 4-cm-long deepithelialized small bowel segment seeded with autologous cells, which was retubularized transversely (Monti) in one mongrel dog. Successful outcomes were reported only in the last set, but bowel mucosa regeneration was noticed in the histology [29].

Ureteral regeneration has also been evaluated without *in situ* implantation of the scaffolds. Zhang *et al.* implanted an 8 Fr Silastic tube in the peritoneal cavity of female beagles. Within 3 weeks after implantation, the tubes had been completely encapsulated by a tubular tissue capsule. Histological analysis showed transversely arranged myofibroblasts embedded in homogenous collagen bundles and an outer layer of mesothelial cells. The tissue was everted and was used to replace a 3-cm ureteral segment. At 12 weeks, the urothelial lining, smooth muscle bundles, and surrounding fibrous adventitia became similar to the normal ureteral wall [30]. Matsunuma *et al.* demonstrated successful seeding of the canine decellularized ureteral matrix with the stratified urothelium and bone marrow-derived mononuclear cells using the subcutaneous tissue of nude mice or the omentum of rats as a natural bioreactor [31]. Baumert *et al.* reported successful urothelial regeneration upon a multilayer smooth muscle connective tissue by placing an SIS patch seeded with autologous cells shaped around a silicone drain in the omentum of female pigs [32]. Shi *et al.* evaluated the differentiation potential of human adipose-derived stem cells (hADSCs) into urothelial lineage after seeding in a hybrid polylactic acid collagen scaffold. These scaffolds were implanted subcutaneously in athymic mice for a period of 2 weeks. They reported differentiation of the hADSCs into UCs, which were maintained after the *in vivo* implantation [33]. ADSCs were also reported to differentiate into SMCs after proper induction. Zhao *et al.* used the decellularized Vessel Extracellular Matrix (VECM) from abdominal rabbit aortas. The VECM was seeded with inducted stem cells and replaced with an approximately 3-cm-long ureteral segment. At 16 weeks after implantation, the stratified epithelium and organized muscle bundles were observed that were similar to the native tissue [34]. Fu *et al.* constructed an electrospun composite poly(L-lactic acid)-collagen and examined the outcomes of seeded with UCs versus nonseeded scaffolds after subcutaneous implantation in nude mice. They also tested cell distribution after seeding with regard to the centrifugal or static seeding method. They concluded that this type of scaffold seeded with the centrifugal technique could be used as a biomatrix for UC growth [35]. Xu *et al.* prepared a spiral poly(L-lactic acid) scaffold and implanted it subcutaneously in Wistar rats. The scaffolds were harvested after 1, 2, and 3 weeks, decellularized, and finally seeded with autologous UCs. The entrapped cells grew well and UCs lined up in a continuous layer at all time points. Besides the cytocompatibility, neovascularization was also noticed [36]. Nevertheless, the functionality of these matrices remains to be evaluated.

Clinical implication and future Perspectives

The primary goal of the engineered ureter is to maintain the safe transportation of urine from the kidney to the bladder. The ureter is an active, contractile tissue that generates peristaltic waves and its role is critical in preserving the normal renal function and avoiding the development of hydronephrosis. In the porcine midureter, the propagation velocity of these peristaltic waves is 2.1 – 1.0 cm/s with a length of pressure peak 5.9 – 1.3 cm [37]. The native ureter is composed of two smooth muscle layers, an inner longitudinal and an outer circular, and therefore, the regenerated tissue should exhibit the same anatomic and functional properties as much as possible.

There is a clear difference in the outcome of published studies depending whether a partial or a complete ureteral segment is replaced. This can be justified by the fact that ureteral regeneration warrants cell migration from the original ureter onto and into the scaffold. Regeneration of the entire length and circumference of the scaffolds is challenging since it requires cell migration over a longer distance than in onlay techniques. When increasing the ureteral defect or the implanted scaffold, the cell growth and regeneration of acellular scaffolds decreases. Since current surgical techniques can be used to repair short ureteral defects, ureteral tissue engineering should contribute to the reconstruction of longer lesions.

This issue can be potentially managed by seeding scaffolds with cells or loading them with growth factors. In urethral tissue engineering, cell seeding is critical for the avoidance of stricture formation when collagen scaffolds are used [38, 39]. When acellular tubular scaffolds were evaluated in urethral reconstruction, normal tissue regeneration was only noticed for 0.5-cm-long defects [40]. In a recent study regarding urinary diversion, Geutjes *et al.* reported that UC seeding may not provide any advantage to the development of urothelium [41]. On the other hand, the cytotoxicity of urine and also its negative influence in tissue regeneration has been demonstrated [42]. To protect the cells during ingrowth and tissue remodeling after *in situ* implantation, seeding UCs in the luminal surface might be beneficial (Fig. 1) [43]. In studies where scaffolds are preimplanted into the peritoneal cavity, the toxic effect of urine is absent and further data regarding regeneration after *in situ* implantation would be interesting.

The construction of cell-seeded scaffolds involves cell harvesting, culture, and seeding onto the scaffold. Ideally, the scaffolds should also be tested in a bioreactor for their mechanical properties before implantation to ensure adequate mechanical strength, and thus avoid intra- and postoperative complications. With tensile and flow studies, it is possible to study the cell response to the forces that are normally applied to the ureter. This is a procedure that consumes time, work hours, and also increases the costs. Cell distribution after seeding is another issue

that has to be determined before implantation. Sun *et al.* examined cell ingrowth in collagen and hybrid scaffolds of different collagen concentrations [ranging from 0.3% to 0.8% (w/v)]. They concluded that a hybrid scaffold prepared from 0.4% collagen strengthened with knitting achieved the best cellular distribution [44]. Autologous cells are preferred since their use ensures a minimal inflammatory response and biocompatibility [45]. To obtain these cells, in most cases, an additional surgery is necessary and this can cause additional morbidity. The technique used for seeding cells should provide equal distribution of living cells. Static, dynamic, and spinning seeding methods are most often used currently [46, 47]. The time needed to perform the aforementioned procedures may be a limitation in their use. Consideration should be given to determine whether the advantages of a cellular construct outweigh these disadvantages, the increased cost, and possibly decreased clinical applicability.

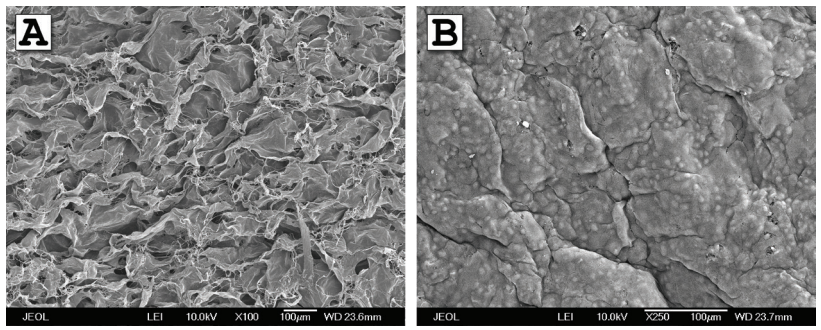

Figure 1. A. Scanning electron microscopy image of the porous surface of a collagen 0.5% scaffold. **B**. Scanning electron microscopy image of the surface of a cell-seeded collagen 0.5% scaffold. A dense layer of cells cover the surface of the collagen scaffold forming a potential barrier between urine and the scaffold.

Growth factors can be an alternative to attract cells inside the ureteral scaffolds. They are known to have a stimulatory effect on various cellular processes, including cell influx, angiogenesis, and proliferation, thus improving the regenerative capacity of the scaffold [48]. Growth factors can be incorporated in biomaterials through a variety of methods, including entrapment within gel matrices, hydrophobic scaffolds, or microparticles, through affinity binding sites and covalent binding to matrices [49]. In ureteral tissue engineering, different growth factors are needed to stimulate different cell populations. The vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) are involved in angiogenesis and blood vessel maturation [50]. The epidermal growth factor (EGF) is known to play a key role in urothelial regeneration [51]. These growth factors are heparin binding growth factors. Heparin can be incorporated in many different biomaterials to create controlled release systems [52]. In a study regarding urethral reconstruction using tubular collagen scaffolds loaded with growth factors (VEGF, FGF-2, and EGF) in a rabbit model, Nuininga *et al.* demonstrated narrowing of the lumen due to urothelium ingrowth. This may be prevented by loading the EGF only in the inner (luminal) side of the scaffold [48].

The insulin-like growth factor-1 (IGF-1) has been explored in an array of tissues, including skeletal and cardiac muscle, nerve, cartilage and bone. Lorentz *et al.* reported an engineered IGF-1 that improved SMC regeneration [53]. The main disadvantage of IGF-1 is that it does not bind to heparin. The main advantage of scaffolds loaded with growth factors is that they can be used off the shelf in urgent cases.

Stem cells (SCs) appear to be a promising area of research in urological regenerative medicine. Their use in tissue regeneration has been tested in bladder augmentation and detrusor regeneration in animal studies and in the treatment of stress urinary incontinence in clinical trials [54, 55]. Studies regarding ureteral regeneration by using SCs are still lacking [56]. Autologous urine-derived SCs exhibit a high expansion rate and capability of differentiating into both urothelial and SMCs [57]. Supplementation of growth factors *in vivo* promoted the survival of urine-derived SCs and their differentiation into muscle cells. Enhancement of nerve regeneration and native cell attraction were also noticed [58]. Issues that need further research are the control of SC proliferation rate, differentiation into the desirable line, and also their behavior in the long term [59].

Collagen currently seems to be the biomaterial of choice in the construction of small diameter tubular scaffolds. Type-I collagen is the most abundant type of collagen in organs and provides strength and structural integrity to tissues. Allogenic collagen, like bovine, exhibits excellent biocompatibility and low immunogenicity in humans [60-62]. Highly purified type-I collagen is commercially available and it is technically feasible to prepare up to 10-cm-long tubular scaffolds. The major disadvantage of collagen scaffolds is their poor physical strength [63]. Chemical crosslinking can enhance their mechanical properties [64]. By experience of our institution, suturability of these scaffolds *in vitro* in a porcine ureteral model is satisfactory and patency is achieved without complications. Another option may be the use of high degradable polymer-collagen scaffolds to increase the physical strength and ease of application. Further research regarding the development of small diameter hybrid scaffolds is required.

The animal model for preclinical studies is also a factor that may affect the outcome of the experiment. The natural algorithm for animal studies—which dictates the use of small animals before proceeding into large animal studies—cannot always be followed strictly as in the case of ureteral tissue engineering. Crossover knowledge from urethral tissue engineering studies can provide data for the regeneration of scaffolds up to 2 cm long [65]. In ureteral reconstruction, a lesion of this length is clinically insignificant since it can be repaired by the available surgical techniques. In longer defects, and to mimic the clinical situation and extrapolate the preclinical data as much as possible, the animal model should have an abdominal and ureteral anatomy analogue to that of a human. This offers the advantages of testing the feasibility of such a procedure, the applicability of the scaffold, and the outcome of the regenerative process. The pig model seems to be the best alternative [66, 67]. Nevertheless, from the ethical perspective, it is important to perform extensive *in vitro*- and ex vivo-related experiments

before animal testing, for example, pressure–flow experiments to characterize the mechanical properties and suturability of the construct.

The ideal scaffold should have a high regenerative capacity, ease of construction, and direct availability in urgent cases. Developing tubular scaffolds for ureteral reconstruction will be of great importance for both the patient and the surgeon. The simplified surgical technique may lead to a less invasive surgery, lower complication rates, and a reduction in health care costs. From this point of view, the evaluation of tubular collagen scaffolds loaded with growth factors seems to be promising. Finally, as in every tissue-engineering application, considerations should be made to bring these new options to applicable techniques in the clinical situation. However, the exact requirements and methods for conducting clinical trials should be defined [68].

Conclusions

Current literature regarding ureteral tissue engineering is lacking evidence as for the determination of a suitable biomaterial. Furthermore, progresses in regenerative medicine, like cell-seeded scaffolds, scaffolds loaded with growth factors, or the use of SCs have not been efficiently evaluated in ureteral reconstruction yet. Further, preclinical research is required to develop a suitable scaffold and improve the tissue- engineering applications for this domain of urological regenerative medicine.

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Chapter 3

Recent advances in ureteral tissue engineering

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Abstract

Reconstruction of long ureteral defects often warrants the use of graft tissue and extensive surgical procedures to maintain the safe transport of urine from the kidneys to the urinary bladder. Complication risks, graft failure related morbidity and the lack of suitable tissue are major concerns. Tissue engineering might offer an alternative treatment approach in these cases, but ureteral tissue engineering is still an underreported topic in current literature. In this review, the most recent published data regarding ureteral tissue engineering are presented and evaluated, with a focus on cell sources, implantation strategies and (bio)materials.

Introduction

Advances in endourology led to an increase in the number of ureteroscopies and nephroscopies during the last two decades worldwide. The complications of these procedures are often underreported, possibly due to lack of early recognition or short term postoperative follow-up [1-3]. Strictures after ureteroscopy occur with an estimated frequency up to 3.5%, which is more often than avulsion or major perforation [4-6]. In around 1% of the gynaecological procedures a ureteral injury occurs, which is estimated to account for up to 73% of all ureteral injuries [3, 7, 8]. In addition to these iatrogenic injuries, trauma can result in ureteral damage. A large retrospective analysis in the United States showed that 2,6% of all urogenital traumas involved the ureter between 2002 and 2006 [9]. The specific anatomic characteristics of the ureter, such as the segmental vascular supply, can be easily damaged, and the lack of native tissue limits surgical ureteral reconstruction. To repair the long ureteral defects, where an end-to-end anastomosis is not feasible for the urologist, several techniques have been introduced such as a ureteroneocystostomy, a Boari flap, ileal interposition and renal autotransplantation. Ultimately, when surgical expertise is not available or the aforementioned techniques do not succeed, an undesirable nephrectomy is the only option [10, 11].

Tissue engineering might offer new treatment approaches in ureteral reconstruction to optimize the outcome in complicated cases, which currently have complication rates up to 25% [12]. The number of published manuscripts dealing with tissue engineering applications of the urinary system is quite extensive, particularly for urinary bladder reconstruction, but the number of research groups that focus on tissue engineering of the ureter is limited, which suggests that the development of an artificial construct suitable for ureteral reconstruction is challenging. Additionally, there might be less incentive to investigate tissue engineering approaches as the incidence of long ureteral injuries is lower than urethra or bladder injuries.

Ureteral tissue engineering

In a recent review on ureteral tissue engineering [13] it was demonstrated that the evidence in the literature was inconclusive about the optimal tissue engineering approach to treat long ureteral injuries. Furthermore, compared to other parts of the urogenital tract, very few tissue engineering studies were performed. Most studies focused on tubular(ized) small intestinal submucosa (SIS) without cellular pre-seeding [14-20]. Other materials included collagen [21, 22] and Gore-Tex [23, 24]. In general, collagen and SIS, but not Gore-Tex, were capable of facilitating some degree of urothelium and smooth muscle regeneration. However, fibrosis occurred in most cases.

In this review we present recent developments in ureteral tissue engineering and discuss currently used materials, construct design, cell sources, and implantation techniques. In addition to the recent literature reviews on ureteral tissue engineering, a Medline search was performed for papers published in the last 3 years using a previously published tissue engineering filter [25] combined

with the MeSH term ureter. An overview of the recent studies is presented in table 1 in which we focused on the early post implantation complications and the presented solutions.

Ureteral defect repair

To study the effect of a tissue engineered construct on the regeneration of the ureter, it is imperative to test the constructs in a ureteral defect model. Nevertheless, in three recent studies the authors refrained from implantation of tissue engineered constructs in an induced ureteral defect model. Instead, the authors performed subcutaneous implantations in rats (Xu, *et al.* [26]) and mice (Shi, *et al.* [27] and Fu, *et al.* [28]). While these studies showed the potential of pre-implantation for ureteral replacement, information about the behavior of the construct as ureteral replacement is lacking. In the intracorporeal environment constructs are exposed to the toxic effects of urine and various mechanical forces [5]. All three studies used a similar spiral PLLA stent as the backbone of their construct. Xu, *et al.* [26] implanted the spiral PLLA stents in the subcutis and used the body as a natural bioreactor to generate a tissue fleece around the stent. The newly formed tissue was then decellularized and re-seeded with primary urinary bladder urothelial cells. Cell proliferation was similar compared to SIS. Shi, *et al.* [27] and Fu, *et al.* [28] combined the same PLLA stent with electrospun collagen to improve cell attachment and cell proliferation. Before subcutaneous implantation in athymic mice, the final constructs were seeded with human adipose derived stem cells (hADSC) (Shi, *et al.* [27]) or human urothelial cells (hUC) (Fu, *et al.* [28]). Both authors were able to detect viable human cells 2 weeks post-implantation, demonstrating that the cells could survive the procedure. These studies solely indicate that the subcutis might be a suitable pre-implantation site to generate a tubular pre-vascularized autologous tissue, which may prevent fibrosis when attempting to repair the ureter.

Authors	*Animal model*	*Biomaterial*	*Cell Seeded*	*Length*	*Tech-nique*	*Outcome*
Xu, *et al.[26]*	Rats (M)	PLLA	No	0.9 cm	S,T	I_1, V
Shi, *et al.[27]*	Mice (F)	PLLA, Collagen	hADSC	-	S,T	hUC
Fu, *et al.[28]*	Mice (M)	PLLA, Collagen	hUC	1.0-1.5 cm	S,T	hUC
Zhang, *et al.[29]*	Dogs (F)	Autologous graft	No	3.0 cm	P,T	UC, SMC, V
Salehipour, *et al.[30]*	Dogs (M)	AM	No	3.0 cm	T*	L, H, F, I_2
Zhao, *et al.[31]*	Rabbits (F)	VECM	ADSC	3.0 cm	T	UC, SMC
Liao, *et al.[32]*	Rabbits (M)	BAM	MSC, SMC	4.0 cm	P, T*	I_1,UC, SMC
De Jonge and Simaioforidis, *et al.(unpublished)*	Pigs(F)	Collagen	UC, SMC	5.0cm	T	UC, SMC, L, F, H

Table 1. Recent ureteral tissue engineering studies. **PLLA**, Poly(L-lactic acid); **AM**, Amniotic membrane; **VECM**, Vessel extracellular matrix; **BAM**, bladder acellular matrix; **(h)ADSC**, (human) adipose derived stem cell; **MSC**, Mesenchymal stem cell; **(h)UC**, (human) urothelial cell; **SMC**, Smooth muscle cell; **S**, subcutaneous implantation; **P**, Pre-implantation; **T**, Tubular; **T***, Tubularized; **I**, Inflammation (I_1: mild, I_2: severe); **V**, Vascularization; **F**, Fibrosis; **H**, Hydronephrosis; **L**, Urine leakage.

Cell sources

The use and necessity of cell seeding of tissue engineered constructs has been a matter of debate, but once it is considered many options exist. Embryonic stem cells are highly controversial due to their origin and the risk of tumor formation. A safer and less controversial option is the use of autologous cells when available. Tissue biopsies can yield differentiated primary cells or multipotent cells like mesenchymal or adipose derived stem cells. Most early studies in ureteral tissue engineering used bare scaffolds and almost all of them resulted in fibrosis, which may indicate the necessity of cell seeding [14-19, 21-24, 33]. This is supported by previous statements that cell seeding is required for large defects (> 1.0cm from the wound edge) to promote tissue regeneration and to prevent scar formation [34]. In most recent studies, cell seeding or pre-implantation of the scaffolds was explored to improve regeneration [27-29, 31, 32].

Fu, *et al.* [28] used primary urothelial cells, isolated from patients that underwent nephrectomy, which were seeded on spiral PLLA stents and subcutaneously implanted in a nude mouse model for 2 weeks. The grafts resulted in a thin tissue capsule in which the seeded cells were still present and viable. This successful approach is relatively straight forward, albeit time consuming since it takes 4 weeks before ureter reconstruction can be performed; the cells are expanded for 2 weeks, followed by pre-implantation for 2 weeks.

A faster approach would be the implantation of only the cell seeded construct. To investigate this approach we implanted 5.0 cm long highly porous tubular 0,5% type-I collagen constructs to repair a full ureteral defect in 11 female Landrace pigs (unpublished data). In brief, primary urothelial (UC) and smooth muscle cells (SMC) were isolated from porcine urinary bladder biopsies [35]. First, the scaffolds were homogenously seeded with urinary bladder derived SMCs, followed by luminal seeding of urinary bladder derived UC. The right ureter was approached and mobilized through a midline incision and a 5.0 cm segment of the ureter was removed and replaced with an equally sized scaffold. A 6 Fr double-J stent was placed to facilitate urinary flow and animals were followed up to 4 weeks. In 7/11 pigs, abdominal swelling due to urine leakage was observed after 2-3 weeks. The other animals developed strictures and hydronephrosis despite the presence of the stent. Upon analysis it became clear that the urine leakage could be attributed to insufficient mechanical strength of the collagen scaffolds, which resulted in ruptures or dissections of the scaffolds. In the animals where the scaffold remained patent, the scaffold was mostly covered by a single layer of urothelial cells. Extensive neovascularization and some SMC ingrowth was observed (Fig 1). Although the collagen construct with primary urinary bladder cells was suitable for ureteral reconstruction, we can conclude that back-bone biodegradable synthetic materials are needed to bear mechanical loads when attempting to repair an unsupported, mobile organ like the ureter.

One of the major disadvantages of primary cells, especially for urothelium, is that the cells cannot safely be harvested in case of possible malignancies [36]. Also, suitable tissue may not always be available for cell isolation. Therefore, alternative cell sources are being explored with a focus on mesenchymal (MSC) and adipose

derived stem cells (ADSC). These cells can differentiate into multiple cell lineages, including muscle and epithelium, without the risk of tumor formation [37-40]. Additionally, they are associated with anti-inflammatory properties and the capability to produce several cytokines that are associated with normal wound healing [41].

Zhao, *et al.* [31] isolated ADSC from rabbits and differentiated these towards a SMC phenotype before cell seeding and implantation. The cells were seeded on decellularized rabbit abdominal aorta to prepare a vascular extracellular matrix (VECM). Cell seeded scaffolds were used to replace a 3.0 cm long defect of the rabbit ureter. After 16 weeks the defect was characterized by a well-organized muscle layer and stratified urothelium similar to native tissue. Strictures and hydronephrosis were absent. The authors attributed the positive results to the stimulating effect of ADSC on SMC proliferation and differentiation, and the use of a graft containing many natural occurring growth factors [42]. Additionally, Shi, *et al.* [27] showed that human ADSC can survive and maintain their phenotype for at least 2 weeks when implanted subcutaneously in nude mice, showing the possibility to use these cells for their stimulating properties in time. Alternatively, MSC can be used. These cells possess similar properties as ADSC, but are isolated from the bone marrow. The harvesting procedure of these cells is painful, the differentiation potential decreases with age, and the number isolated cells is limited compared to ADSC [43, 44]. Liao, *et al.* [32] used MSC in combination with SMC seeded on bladder acellular matrix (BAM) to repair a 4.0 cm ureteral defect in rabbits. The BAM was seeded with bone marrow derived mesenchymal stem cells (MSCs) on one side and urinary bladder derived SMCs on the other side to create tissue-engineered tubular grafts (TETG). The TETG was tubularized around a catheter and pre-implanted in the omentum of rabbits for 2 weeks. During this pre-implantation, the MSC differentiated and formed a single-cell layered epithelium. Next, the ureteral defect was repaired, where a multilayer urothelium with central neovascularization was observed after 16 weeks. No strictures or hydronephrosis was observed, even though the ureteral catheter was removed 6 weeks post-operatively. Without MSC the ureter repair resulted in scar formation and severe hydronephrosis. The investigators reasoned that the formation of the single layered epithelium during the pre-implantation phase might have protected the surrounding tissue against urine.

These examples show the potential of stem cells as an alternative cell source for ureteral tissue engineering when insufficient donor tissue is available.

Full circumference ureteral defect repair

Major ureteral reconstructions are required when a complete segment of the ureter needs to be replaced. Onlay graft repair is most often impossible and can only be applied in stricture repair. It is therefore not surprising that the majority of the studies focus on the repair of long (relative to the total length of the ureter) defects using tubular or tubularized constructs. Zhang, *et al.* [29] used 3.0 cm long tubular autologous connective tissue that was formed after the implantation of silicon tubes in the peritoneal cavity of dogs. By maintaining one third of the

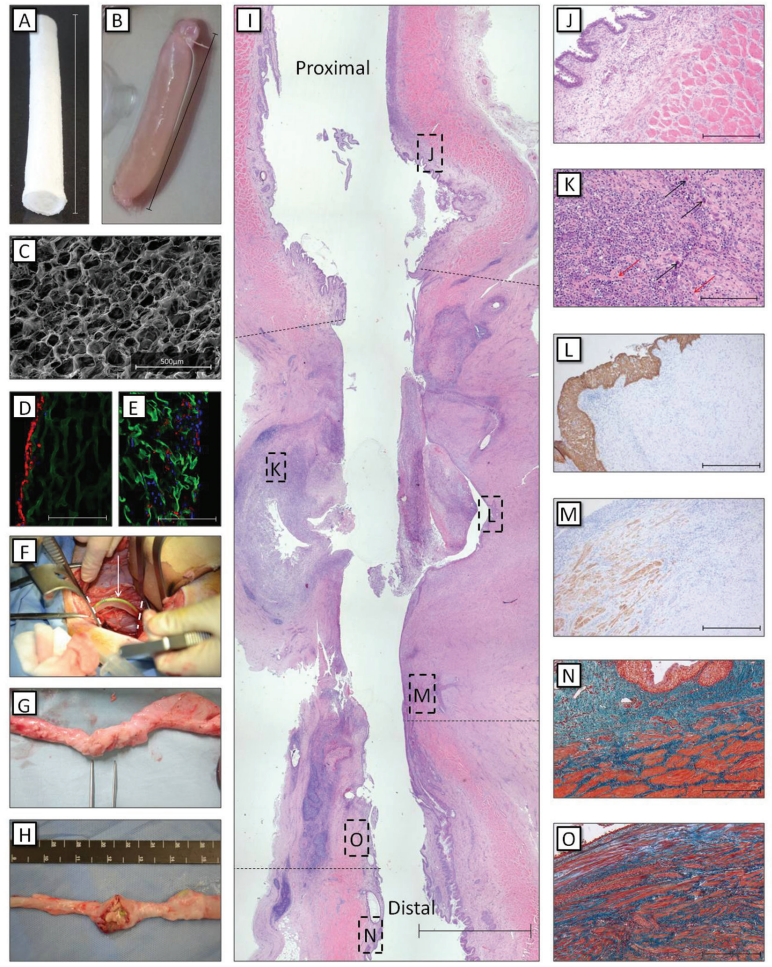

Figure 1. Implantation strategy and outcome after ureteral reconstruction using tubular collagen scaffolds. **A,B**: Macroscopic overview of a tubular 0.5% type-I collagen scaffold (length = 6 cm, Ø = 6 mm). **C**: SEM overview of the scaffold surface, which was highly porous, facilitating cell penetration into the scaffold (scale bar = 500 µm) **D**: Immunofluorescent staining for collagen (green), nuclei (blue) and RCK103 (red) of a cell seeded scaffold. Urothelial cells (RCK103 positive) were lining the scaffold (scale bar = 400 µm). **E**: Immunofluorescent staining for collagen (green), nuclei (blue) and α smooth muscle actin (red) of a cell seeded scaffold. Smooth muscle cells (α smooth muscle actin positive) were found throughout the scaffold (scale bar = 400 µm). **F**: The scaffolds were implanted by end-to-end anastomosis. **G,H**: Ureteral regeneration was evaluated after 1 month. 4 animals showed intact ureters (G), while 7 animals presented with defects or dissections (H). **I**: Histological overview of a regenerating ureter (scale bar = 5 mm). Black dotted lines indicate the anastomosis sites. Specific locations are highlighted (J-O). **J**: Haematoxylin and eosin (HE) staining of the native ureter (scale bar = 400 µm). **K**: Inflammatory response in the regenerating tissue near scaffold remnants (red arrows). Mostly lymphocytes, a few granulocytes and some multinucleated giant cells (black arrows) were observed (scale bar = 200 µm). **L**: Pancytokeratin staining in the middle of the neo-ureter, indicating the presence of (multilayered) urothelium (scale bar = 400 µm). **M**: Smoothelin staining near the anastomosis site, indicating ingrowth and maturation of smooth muscle tissue into the neo-ureter (scale bar = 400 µm). **N**: Masson's trichrome staining of the native ureter (scale bar = 400 µm). **O**: Masson's trichrome staining near the anastomosis site, indicating the ingrowth of new muscle tissue (scale bar = 400 µm).

ureter the investigators kept adequate vascularization and they managed to generate new tissue which was similar to the normal ureter. After 12 weeks, the tubular construct was completely lined by multilayered urothelium, and presented with an organized muscle layer and mucosal folds. While these results are very promising, one has to realize that it is unlikely that one third of healthy ureter is present in the clinical situation, e.g. in case of severe adhesions or prolonged avascularity. It is generally accepted that tissue ingrowth after 1.0 cm becomes increasingly difficult and is likely to be accompanied by fibrosis [34]. By maintaining the ureteral segment, the authors avoided this challenge.

Pre-implantation

The lack of functional urothelium and adequate vascularization may contribute to stricture formation and fibrosis as urine can freely damage the regenerating tissue [5, 45]. Pre-implantation promotes vascularization and helps to maintain the viability of the seeded cells *in vivo*. Different pre-implantation sites have been used for various applications, including the omentum [46, 47], peritoneum [48, 49] and subcutis [49, 50]. Zhang, *et al.* [29] and Liao, *et al.* [32] took advantage of pre-implantation before repairing a ureteral defect. Zhang, *et al.* exploited pre-implantation to create a tubular scaffold from the fibrous capsule which was formed in the peritoneal cavity. Liao, *et al.* [32] used omental pre-implantation as an *in vivo* bioreactor to increase neo-vascularization in the construct. Additionally, it allowed the formation of a one-layer epithelial structure which may protect the construct after implantation in the toxic urine-rich environment.

Ideally, when harvesting the pre-implanted material, the newly formed blood vessels should remain intact. A mobile pre-implantation site close to the ureteral defect repair site, like the greater omentum, might be suitable for this as flaps can easily be mobilized most of the time. Although pre-implantation techniques are promising, it is time consuming and requires a second surgical procedure. Therefore, it may not always be applicable in case of acute problems and unplanned procedures, which is often the case with ureteral trauma.

Decellularized tissue and synthetic polymers

A variety of materials has been used as scaffolding material. Most studies used decellularized tissues as opposed to "man-made" scaffolds in the past decades. The advantages of decellularized tissues include preservation of the native tissue architecture and inclusion of tissue specific growth factors and other signaling molecules [42]. In the past, SIS has been the decellularized tissue of choice. The results, however, were not optimal in ureteral tissue engineering. Recently, Salehipour, *et al.* [30] used amniotic membrane (AM), which is known for its anti-inflammatory properties, as a biomaterial to reconstruct long ureteral defects. In dogs a 3.0 cm segment of the ureter was replaced by tubularized decellularized AM. Two out of seven animals died due to urine leakage and another animal showed severe hydronephrosis, acute and chronic inflammation, and the formation of granulation tissue. The other animals presented with mild pelvicaliectasis and fibrosis of the reconstructed segment with lymphatic and granulocytic infiltration. Where Koziak, *et al.* [51] showed encouraging results when AM was used as an onlay graft in 2007, the authors of this study concluded that AM did not act as a favorable material when used in full defects. This result was similar to a previous study by Osman, *et al.* in 2004 [33]. Decellularized blood vessels and bladder acellular matrix have recently been used with promising results, but these results may also be caused by the use of stem cells and pre-implantation techniques, something that was not done in combination with SIS for the ureter [31, 32].

Besides decellularized tissues, "man-made" scaffolds can be used. The advantage of these scaffolds is a higher degree of plasticity, good mechanical properties, and they are well defined. Most materials can be prepared in any shape (e.g. flat, film or tube) or size, and different proteins and bioactive molecules can be added as demonstrated in the recent publications using spiral PLLA stents in combination with collagen and our tubular collagen scaffolds. When improvements such as increased mechanical strength are required, these man-made scaffolds can easily be tailored compared to decellularized tissue.

Animal models

In recent ureteral replacement animal studies rabbits [31, 32], dogs [29, 30] and in our case pigs (unpublished), were used, while subcutaneous implantation studies were performed in rats [26] or mice [27, 28]. The pig is the preferred model because the abdominal anatomy of pigs and humans is similar [52, 53]. Nevertheless, the lack of recent pig studies might be associated with the high incidence of fibrosis and fast growth of the animal, as mostly fast growing young pigs are used. This may influence tissue regeneration and cause mechanical stress on the tissue constructs. The ideal animal should have a similar size and abdominal anatomy as humans, be fully grown, and have similar wound healing characteristics. Potential candidate animals include goats, sheep, cattle and horses. In general, randomized controlled trials preceded by extensive toxicity studies are required before a new technique is widely used in the clinic. However, in tissue engineering it is unethical to perform safety studies in healthy patients and there is often a lack of golden standard treatments. Therefore, choosing the right animal models is critical to predict the expected clinical outcome as good as possible [54].

Conclusions

Ureteral reconstruction should focus on the maintenance of safe urine transport from the kidney to the bladder. Fast development of a vascular system, a functional smooth musculature, and a urothelial barrier are critical for the success of constructs as the lack of these layers may result in strictures and hydronephrosis, even when stents are used. In the past few years, clear advancements have been made in ureteral tissue engineering. Specifically, the cell source, implantation techniques and new biomaterials have improved the tissue engineering of the ureter. Decellularized tissues or scaffolds with added natural proteins and other molecules may perform better than simple scaffolds, however, these were only studied in the context of stem cells. Despite these advancements, published

research in the area of ureteral tissue engineering is scarce. Many recent studies do not address the behavior of the constructs in a ureteral replacement setting. To increase our knowledge on the effect of different materials, cells sources and implantation techniques, future studies should attempt to repair a full ureteral defect. Current literature suggests that the use of mesenchymal and adipose derived stem cells, seeded on any type of mechanically suitable bioactive material, is optimal for ureteral regeneration. In addition, pre-implantation of these constructs in the omentum may improve the final outcome by increasing vascularization and triggering stem cell differentiation. However, when using the body as an *in vivo* bioreactor, the long incubation time may be problematic in ureteral repair. Finally, different pre-clinical animal models should be evaluated to prevent species-related result bias prior to commencing clinical trials.

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Chapter 4

Tissue engineering of the urethra: a systematic review and meta-analysis of pre-clinical and clinical studies

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Abstract

Context: Urethra repair by tissue engineering has been extensively studied in laboratory animals and patients, but is not routinely used in clinical practice.
Objective: To systematically investigate pre-clinical and clinical evidence of the efficacy of tissue engineering for urethra repair in order to stimulate translation of pre-clinical studies to the clinic.
Evidence Acquisition: A systematic search strategy was applied in PubMed and EMBASE. Studies were independently screened for relevance by two reviewers, resulting in 80 pre-clinical and 23 clinical studies of which 63 and 13 were selected for meta-analysis to assess side-effects, functionality, and study completion. Analyses for pre-clinical and clinical studies were performed separately. Full circumferential and inlay procedures were assessed independently. Evaluated parameters included seeding of cells and type of biomaterial.
Evidence Synthesis: Meta-analysis revealed that cell seeding significantly reduced the probability of encountering side-effects in pre-clinical studies. Remarkably though, cells were only sparsely used in the clinic (4/23 studies) and showed no significant reduction of side-effects. In 21 out of 23 clinical studies, decellularized templates were used, while in pre-clinical studies other biomaterials showed promising outcomes as well. No direct comparison to current clinical practice could be made due to the limited number of randomized controlled studies.
Conclusion: Due to a lack of controlled (pre-)clinical studies, the efficacy of tissue engineering for urethra repair could not be determined. Meta-analysis outcome measures were similar to current treatment options described in literature. Surprisingly, it appeared that favorable pre-clinical results, i.e. inclusion of cells, were not translated to the clinic. Improved (pre-)clinical study designs may enhance clinical translation.
Patient Summary: We reviewed all available literature on urethral tissue engineering to assess the efficacy in pre-clinical and clinical studies. We show that improvements to (pre-)clinical study design is required to improve clinical translation of tissue engineering technologies.

Introduction

Congenital birth defects of the urethra, such as hypospadias (1 in every 300 births) [1, 2], and acquired urethral abnormalities, such as urethral strictures (1 in every 1,000 men >65 years of age [3]), represent major clinical entities. Treatment usually involves a surgical procedure with risk of (recurrence of) strictures or fistula requiring additional care or reintervention. Whenever possible, local tissue flaps or stricture resection in combination with end-to-end anastomosis are used for urethra reconstruction [4, 5]. Generally, two surgical approaches exist for urethral reconstruction: partial replacements using onlay or inlay techniques or the full circumferential procedure, which is used in rare cases with significant urethral scarring or lichen sclerosis. Depending on patient and local factors, procedures can be performed as one-stage procedure or as planned multistage procedure [3].

Autologous tissue transplantation such as buccal mucosa or free skin grafts are the standard treatments [6-9]. However, due to the limited quantity of available donor tissue, accompanying donor site morbidity (16 to 32% for buccal mucosa grafts) and complications (e.g. recurrences or infections), alternative treatment options are needed to improve long-term outcome [10]. Tissue engineering may overcome some of the aforementioned disadvantages by providing a temporary template to guide tissue regeneration [11]. In general, tissue engineered templates include decellularized tissue or de-novo prepared materials from natural or synthetic origin [12-14]. Templates can be seeded with (stem) cells from the patient prior to implantation. These cells may stimulate tissue remodeling by excreting cytokines and growth factors and contributing to cellular population of the template [15, 16].

Despite the potential of tissue engineering shown in in vitro research and pre-clinical studies, clinical translation is limited. To improve translation, an evidence-based approach, such as systematic reviews, can be applied when designing new tissue engineering strategies. This will avoid unnecessary replication of studies and will help to select the most optimal experimental design and model. We are the first to perform a comprehensive systematic review of evidence for the efficacy of urethral tissue engineering in pre-clinical and clinical studies. A meta-analysis was used to compare different experimental designs based on clinically relevant outcomes. This systematic review aims to improve the translation of urethral tissue engineering from bench to bedside.

Evidence acquisition

Literature search

To identify all available studies on urethral tissue engineering published and indexed up until June 1, 2016, a systematic search strategy was applied in PubMed (Appendix 1) and Embase (via OvidSP; Appendix 2). This strategy combined a tissue engineering search component containing synonyms for tissue engineering related terms [17] with a customized search component for urethra or urethra-related diseases. MeSH terms and EMTREE terms were used in PubMed and Embase, respectively, together with separate words or word combinations in title

or abstract. Next, either an animal filter designed by Hooijmans et al. (PubMed) [18] or de Vries et al. (Embase) [19] was applied (Appendix 1 and 2, search component 3A) or a custom filter for clinical studies (Appendix 1 and 2, search component 3B). In addition, retrieved reviews were screened for primary studies not found using the search strategy. Clinical studies found during animal search strategy were marked and screened for relevance and vice versa.

Study selection

Duplicates in retrieved articles were removed in EndNote (Version X7.2, Thomson Reuters). Studies were assessed independently by LV and PdJ. First, clearly irrelevant studies were excluded based on title. Next, titles and abstracts of the remaining articles were screened for relevance in Early Review Organizing Software (EROS, Buenos Aires, Argentina, www.eros-systematic-review.org) using the following exclusion criteria: 1) no urethra, 2) no tissue engineering, 3) no animals or patient, 4) no primary study. A study was considered to be about tissue engineering when a processed template was used. Studies on tissue transplants or reconstructive surgery without the use of a template or without a urethra defect were excluded. Of the remaining studies full texts were screened using the same exclusion criteria. Articles not available as full text were excluded at this stage. No language restrictions were applied in the screening phase. If necessary, Google translate was used. Retrieved studies from search updates were directly screened in Endnote according to the same principles. In all stages of the selection process, discrepancies between reviewers were discussed until consensus was reached.

Study characteristics

From all included studies, general information (author, year) and study characteristics (age range of patients, animal species, sex, surgical procedure, type of biomaterial, type of cells) were extracted and listed in Table 1 for pre-clinical studies and Table 2 for clinical studies. For languages other than English, German and French, Google Translate was used to retrieve study characteristics.

Extraction outcome data

Three outcome measures were used to evaluate study outcome: 1) incidence of side effects, e.g. strictures, stenosis, fistulae, and infections, 2) functionality, defined as the ability to void with continence, and 3) study completion, for animals defined as survival until predetermined endpoint and for clinical studies as available for follow-up or no additional urethroplasty required. Only English, German and French studies were considered for quality assessment and meta-analysis. When critical information needed (e.g. surgical procedure or number of animals/patients) was incomplete, studies were excluded. As only two studies used rats these were also excluded at this stage.

Table 1: Study characteristics of all 80 pre-clinical studies sorted on inclusion in meta-analysis, surgical procedure and biomaterial.

	Author	Year	Animal model	# of animals	Sex	Surgical Procedure (defect length in mm)	Biomaterial (category)	Type of added cells	Quality Assessment + Meta-analysis
1	Feng, C.	2011	Rabbit	28	M	Inlay (15)	Acellular corpus spongiosum, porcine (D)	Autologous corporal SMC's and lingual keratinocytes	Yes
2	Ayyildiz, A.	2006	Rabbit	10	M	Inlay (5)	Alloderm® + acellular pericardium, bovine (D)	-	Yes
3	Chen, F.	1999	Rabbit	10	M	Inlay (10)	BAM, porcine (D)	-	Yes
4	Chun, S.Y.	2015	Rabbit	10	M	Inlay (20)	BAM, porcine (D)	Autologous minced urethral muscle and urothelial tissue	Yes
5	Sayeg, K.	2013	Rabbit	18	M	Inlay (35)	BAM, porcine (D)	Autologous bladder SMC's	Yes
6	Huang, J.W.	2014	Rabbit	30	M	Inlay (15)	BAM, rabbit (D)	-	Yes
7	Li, C.	2008	Rabbit	24	M	Inlay (20)	BAM, rabbit (D)	Autologous oral keratinocytes	Yes
8	Li, C.	2013	Rabbit	27	M	Inlay (20)	BAM, rabbit (D)	Autologous oral kereatincytes and TGF-β siRNA transfected fibroblasts	Yes
9	Li, H.	2014	Rabbit	36	M	Inlay (20)	BAM, rabbit (D)	Epithelial-differentiated rabbit adipose-derived stem cells	Yes
10	Wang, F.	2014	Rabbit	12	M	Inlay (10)	Denuded amnion, human (D)	Rabbit urothelial cells	Yes
11	Kajbafzadeh, A.M.	2014	Rabbit	12	M	Inlay (5)	Preputial acellular matrix, human (D)	-	Yes
12	Kawano, P.R.	2012	Rabbit	24	M	Inlay (10)	SIS, 1- and 4 –layer, porcine (D)	-	Yes
13	Guo, H.	2015	Rabbit	24	M	Inlay (20)	SIS, porcine (D)	Autologous keratinocytes and TIMP siRNA transfected fibroblasts	Yes
14	Kropp, B.P.	1998	Rabbit	8	M	Inlay(10)	SIS, porcine (D)	-	Yes
15	Rotariu, P.	2002	Rabbit	7	M	Inlay (25)	SIS, porcine (D)	-	Yes
16	Villoldo, G.M.	2013	Rabbit	15	M	Inlay(10)	SIS, porcine (D)	-	Yes
17	Shokeir, A.	2003	Dog	21	M	Inlay (30)	UAM, dog (D)	-	Yes
18	Huang, J.W.	2015	Rabbit	30	M	Inlay (20)	Cellulose (N)	Rabbit Lingual keratinocytes	Yes
19	Xie, M.	2013	Dog	10	F	Inlay (50)	Silk fibroin (N)	Autologous oral keratinocytes and fibroblasts	Yes
20	Xie, M.	2013	Dog	9	F	Inlay (30)	Silk fibroin (N)	Dog urothelial cells	Yes
21	Sun, D.	2014	Rabbit	21	M	Inlay (5)	Subcutaneous implanted autologous minced muscle (N)	Human umbilical cord MSC's	Yes
22	Xu, Y.	2014	Rabbit	21	M+F	Inlay (5)	Subcutaneous implanted autologous muscle microsomes (N)	Human umbilical cord MSC's	Yes
23	Zhang, K.	2015	Rabbit	12	M	Inlay (20)	P(LA/CL) + type I collagen in combination with ICG-001 (Wnt-pathway inhibitor) (S)	Rabbit bladder urothelial cells	Yes
24	Wang, D.J.	2015	Rabbit	24	M	Inlay (5)	Polylactid acid (S)	Rabbit AdSC's	Yes
25	Kelami, A.	1971	Dog	10	M	Inlay (30)	PTFE (S) + lyophilized dura, human (D)	-	Yes
26	Chung, Y.G.	2014	Rabbit	8	M	Inlay (20)	Silk fibroin (N) + SIS (D)	-	Yes
27	Lv, X.	2016	Rabbit	18	M	Inlay (15)	Silk-Keratin-Gelatin-Calcium peroxide (N) + SIS, porcine (D)	-	Yes
28	Nuininga, J.E.	2003	Rabbit	18	M	Inlay (10)	SIS, 1 - and 4 - layer, porcine (D) + Type I collagen (N)	-	Yes
29	Zhang, Q.	2008	Rabbit	12	M	Full (10)	Acellular amnion, human (D)	Homologous endothelial progenitor cells	Yes
30	Parnigotto, P.P.	2000	Rabbit	12	M	Full (10)	Acellular aorta, rabbit (D)	-	Yes
31	DeFilippo, R.E	2002	Rabbit	24	M	Full (10)	BAM (D)	Autologous bladder SMC's and urothelial cells	Yes
32	El-Tabey, N.	2012	Dog	14	F	Full (30)	BAM (D)	Autologous bladder SMC's and urothelial cells	Yes
33	Wang, J.H.	2013	Rabbit	18	M	Full (30)	BAM with polylactid acid – glycolic acid with VEGF (D)	-	Yes
34	DeFilippo, R.E.	2015	Rabbit	15	M	Full (30)	BAM, porcine (D)	Autologous bladder SMC's and urothelial cells	Yes
35	Dorin, R.P	2008	Rabbit	12	M	Full (5-30)	BAM, porcine (D)	-	Yes
36	Orabi, H.	2012	Dog	21	M	Full (60)	BAM, porcine (D)	Autologous bladder SMS's and urothelial cells	Yes
37	Fu, Q.	2007	Rabbit	18	M	Full (15)	BAM, rabbit (D)	Autologous foreskin epidermal cells	Yes

Table 2: Study characteristics of all 23 clinical studies sorted on inclusion in meta-analysis, surgical procedure and biomaterial.

	Author	Year	Number of patients	Sex	Age range	Number of patients with prior surgery	Surgical procedure (defect length in mm)	Biomaterial (category)	Availability biomaterial	Type of added cells	Quality Assessment + Meta-analysis
1	Atala, A.	1999	4	M	4 – 20 y	4	Inlay (50-150)	BAM, human (D)	Exp	-	Yes
2	El-Kassaby, A.W.	2003	28	M	22 – 61 y	Unknown	Inlay (15-160)	BAM, human (D)	Exp	-	Yes
3	El-Kassaby, A.W.	2008	15	M	21 – 59 y	9	Inlay (20-180)	BAM, human (D)	Exp	-	Yes
4	Le Roux, P.J.	2005	9	M	15 – 56 y	5	Inlay (10-50)	SIS, porcine (D)	Com	-	Yes
5	Palminteri, E.	2006	20	M	20 – 74 y	16	Inlay (20-80)	SIS, porcine (D)	Com	-	Yes
6	Donkov, I.I.	2006	9	M	26 – 45 y	5	Inlay (40-60)	SIS, 4-layer, porcine (D)	Com	-	Yes
7	Fiala, R.	2007	50	M	45 – 73 y	Unknown	Inlay (40-140)	SIS, 4-layer, porcine (D)	Com	-	Yes
8	Orabi, H.	2013	12	M	1.5 – 15 y	3	Inlay (15-35)	SIS, 4-layer, porcine (D)	Com	-	Yes
9	Xu, Y.M.	2013	28	M	2 – 69 y	28	Inlay (35-70)	SIS, 4-layer, porcine (D)	Com	-	Yes
10	Hauser, S.	2006	5	M	61 – 80 y	5	Inlay (35-100)	SIS, 1- and 4 –layer, porcine (D)	Com	-	Yes
11	Osman, N.I.	2014	5	M	36 – 66 y	4	Inlay (?)	De-epidermised dermis, human (D)	Exp	Autologous buccal mucosa-derived keratinocytes and fibroblasts	Yes
12	Fossum, M.	2012	6	M	14 -44 m	Unknown	Inlay (?)	Acellular skin, human (D)	Exp	Autologous urothelial cells	Yes
13	Raya-Rivera, A.	2013	5	M	10 – 14 y	2	Full (40-60)	Polyglycolic acid and poly-(lactide-co-glycolide acid) (S)	Exp	Autologous bladder SMC's and urothelial cells	Yes
14	Bhargava, S.	2008	5	?	Unknown	5	Inlay (?)	De-epidermised dermis, human (D)	Exp	Autologous buccal mucosa-derived keratinocytes and fibroblasts	No #
15	Carpenter, C.P.	2012	1	M	68 y	1	Inlay (25)	Alloderm® (D)	Com	-	No‡
16	Kim, J.Y.	2005	1	M	48 y	0	Inlay (40)	Alloderm® (D)	Com	-	No‡
17	Mantovani, F.	2003	1	M	72 y	1	Inlay (?)	SIS, porcine (D)	Com	-	No‡
18	Lin, J.	2005	16	M	18 – 46 y	Unknown	Full (?)	Acellular skin, human (D)	Exp	-	No (CN)†
19	Villavicencio, H.	1989	22	?	28 – 80 y	Unknown	Inlay + full (?)	Lyophilized dura, human (D)	Exp	-	No (ES)†
20	Glybochko, P.	2015	1	M	64 y	Unknown	Unclear (?)	Acellular artery, human (D)	Exp	Autologous buccal mucosa-derived keratinocytes	No (RU)†
21	Yang, W.Z.	2011	8	M	4 – 23 y	0	Unclear (?)	Acellular skin human (D)	Exp	-	No*
22	Mantovani, F.	2002	5	M+F	70 – 79 y	Unknown	Unclear (?)	SIS, porcine (D)	Com	-	No (IT)†
23	Li, P.	2009	8	M	8 – 36 m	Unknown	Unclear (25-45)	Gelating sponge (N)	Exp	-	No (CN)†

Excluded from meta-analysis because same patients were included in long-term follow-up study by Osman *et al.*, 2014 (#11). ‡ Excluded from meta analysis due to case study. † Excluded from meta-analysis due to language restrictions defined in section 2.5. * Excluded from meta analysis due to unclear surgical procedure. ? = unclear, BAM = bladder acellular matrix, CN = Chinese, Com = Commercial availability, D = decellularized, ES = Spanish, Exp = Experimental availability, IT = Italian, MSC = mesenchymal stem cells, N = natural, RU = Russian, S = synthetic, SIS = small intestinal submucosa, SMC = Smooth muscle cell.

Quality assessment

Due to the non-randomized, non-controlled nature of most pre-clinical and clinical studies, no standard risk of bias analysis could be performed as validated tools are unavailable for these types of studies. Instead, overall quality was independently scored by PdJ and LV based on the reporting of specific key information (Fig. 2, Results section). Discrepancies were discussed until agreement was reached.

Meta-analysis

The following main research question was considered: "What is the evidence for the efficacy of urethral tissue engineering in pre-clinical and clinical studies?" Sub-questions included the effects of the addition of (stem) cells to the template, the type of biomaterial, as well as potential differences between animal species on the separate outcome measures. Analyses for pre-clinical and clinical studies were conducted separately, as were full circumferential and inlay procedures. Statistical analyses were performed with SAS/STAT® software v9.2 for Windows, copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA.

Pre-clinical studies

The following pre-clinical data were extracted for all available time points per study: the total number of animals as well as the number of animals without side effects, with functionality, and alive at the study endpoint. Time points were categorized in three periods: 0-4 weeks, 5 11 weeks and 12 weeks or longer.

Per study, the probability of response (e.g. having no side effects) with a corresponding 95% exact (Clopper-Pearson) confidence interval (CI) was estimated per outcome. An additive random-effects logistic meta-regression model was fitted by means of a generalized linear mixed model approach. The number of responding animals out of the total was used as outcome parameter. In addition, the following independent parameters were used: treatment (combining the addition of cells and the type of biomaterial) and animal species. Random effects for study and for treatment grouped by study, were added. The Akaike Information Criterion [20] showed that models based on combined study data were preferable to models based on the period data (period as factor), therefore all time points per study were combined. When possible, the maximum likelihood approach with adaptive quadrature was used as estimation method. If this did not converge, the maximum likelihood with the Laplace approximation was applied. The resulting estimated odds were backtransformed into percentages and corresponding 95% CIs. In addition, the marginal effects of the treatments were estimated by combining the estimated percentages for rabbits and dogs, including 95% logit-based CIs, as described by Zou [21]. P-values were based on these confidence intervals.

Clinical studies

For the analyses of the clinical outcomes, the following data per study were extracted: total number of patients, and numbers of patients without side effects, with functionality, and completing the study. No separate time points were analyzed in the human studies. For each study, the probability of response with corresponding 95% exact CIs was estimated per outcome. Due to limited study diversity, meta-regression models similar to pre-clinical studies were only fit for inlay repair and biomaterial type 'decellularized'. A compound symmetry random effect was added for the addition of cells, grouped by study. Estimated odds from meta-regression were backtransformed into probabilities and corresponding 95% CIs.

Evidence synthesis

Literature search and screening

Figure 1A and B show the results of the literature search and screening of collected studies. After the search, 1,524 unique pre-clinical and 5,361 unique clinical studies were identified. During title and abstract screening of these studies, 1,349 and 5,282 were excluded, respectively. After full text screening, 80 pre-clinical studies and 23 clinical studies were included in the study characteristics table (see section 3.2). Only 63 pre-clinical and 13 clinical studies were eligible for the quality assessment (section 3.3) and meta-analysis (section 3.4).

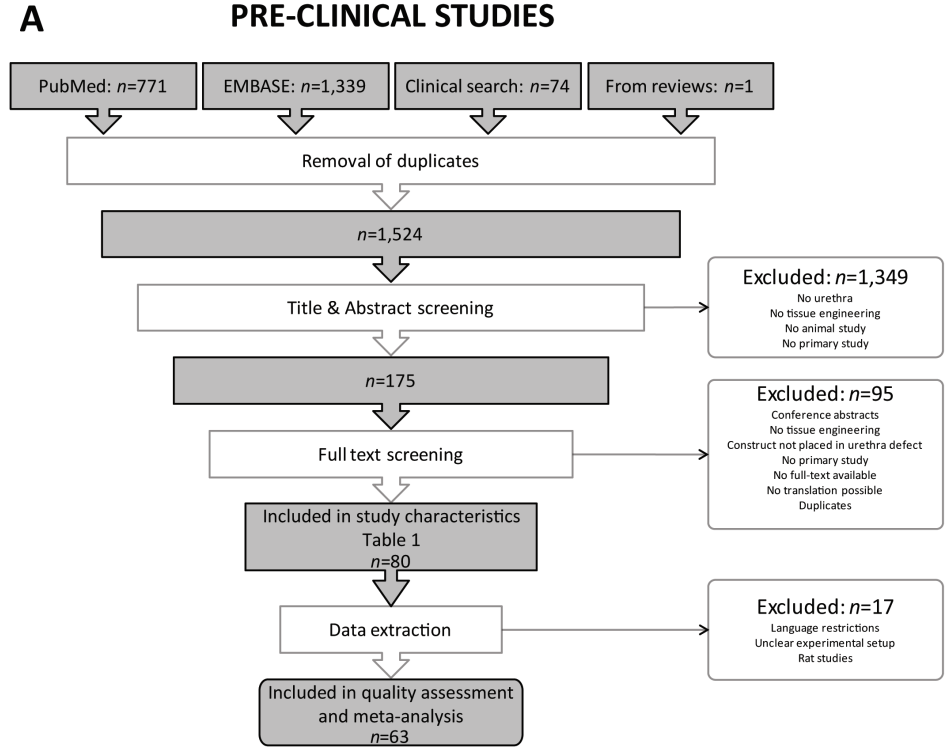

Figure 1A. Flowchart of search and screening process of pre-clinical studies. The search was updated until June 1, 2016.

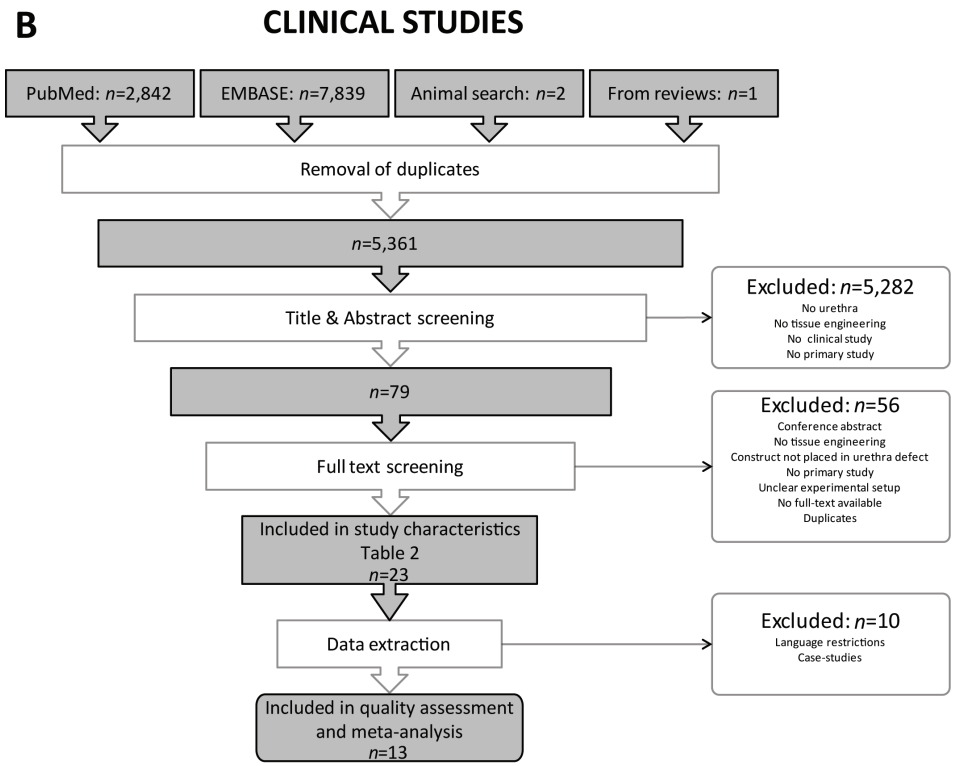

Figure 1B. Flowchart of search and screening process of clinical studies. The search was updated until June 1, 2016.

Study characteristics

Pre-clinical studies

Pre-clinical study characteristics are summarized in Table 1 (see Appendix 3 for references of listed studies). Only three animal species, rabbits (59/80), dogs (19/80) and rats (2/80) were used, which were predominantly males (72/80). Full circumferential repair was investigated in 41 studies, inlay repair in 30 studies, both methods in three, while the procedure was unclear in the remaining studies (6/80). In dogs, primarily full defect repairs were performed (14 full vs. 4 inlay), while in rabbits both inlay (25) and full repairs (26) were employed.

Due to the wide variety of materials used, they were categorized into three categories: decellularized templates (46/80), de novo prepared templates from natural materials (18/80), and de novo prepared templates from synthetic materials (12/80). Four (4/80) studies used multiple material types in

different groups and these were assessed separately in the meta-analysis. Synthetic materials were almost exclusively used for full repair (10 full vs. 3 inlay). Cells were incorporated into templates in 34 studies, of which bladder smooth muscle cells (SMCs) and urothelial cells were mostly used (13/34), followed by keratinocytes and fibroblasts from oral tissue (6/34) or a combination thereof (2/34), foreskin epidermal cells (2/34) and omental mesothelial cells (1/34). Stem cells, mostly derived from adipose tissue, bone marrow or human umbilical cord, were used in 10 studies.

Clinical studies

Study characteristics of clinical studies are listed in Table 2 (see Appendix 4 for references of listed studies). Clinical studies were performed with males, except for one study (Table 2, #22). From 23 studies, 16 used an inlay approach, two a full circumferential procedure, one used both approaches, while in four studies the procedure was unclear. The majority of studies (21/23) used decellularized templates, while natural and synthetic templates were both used once. Four studies used cell-seeded templates; 2/23 buccal mucosa keratinocytes and/or fibroblasts and 2/23 bladder SMCs and/or urothelial cells.

Quality assessment

The quality of reporting was assessed for 63 pre-clinical and 13 clinical studies from which outcome data could sufficiently be extracted for inclusion in the meta-analysis (Fig. 2). Results per study are listed in Appendix 5. Reporting of information regarding included animals/patients, such as species and strain, sex, number of animals/patients, age/weight and patient inclusion criteria, were generally well described.

Overall quality of the experimental setup was poor. Although the different experimental groups were well described, hardly any control groups were present, and randomization and blinding were seldom mentioned in both pre-clinical and clinical studies. Also, clinical study protocols were not published. However, surgical procedure, composition, size and preparation of the implants were clearly described in most studies. Reporting of outcome measures was good for both pre-clinical and clinical studies with respect to the description of outcome measures, follow-up time and side effects. The number of drop-outs was clearly mentioned in clinical studies, but only in half of the pre-clinical studies. For pre-clinical studies, histological sampling location and representativeness of the results were poorly described.

Meta-analysis

Pre-clinical studies

For full circumferential repair (Fig. 3A), the addition of cells significantly reduced the probability of side effects, independent of the type of biomaterial used (p=0.001). Exact point estimates including CI are given in Appendix 6. Regarding the type of biomaterial, when no cells were used, estimates show that synthetic materials had a higher probability for having no side-effects compared to decellularized and natural materials. With cells seeded, estimated probabilities

were similar for all materials. For functionality and study completion, estimated probabilities were similar for all study conditions.

For inlay repair (Fig. 3B), the addition of cells significantly reduced the probability of side effects (p=0.003), albeit less than for full repair. Estimated probabilities were similar for all types of biomaterial regardless of the addition of cells. For functionality and study completion, estimated probabilities were similar for all study conditions. It was impossible to estimate study completion probability per biomaterial as almost all animals survived inlay repair (statistical model did not converge).

Although estimated probabilities for dogs and rabbits were slightly different, differences were not statistically significant. Consequently, the animal species had only marginal influence on outcome (data not shown).

Clinical studies

For clinical studies, a similar meta-analysis was performed (Fig. 3C). Only inlay repair using decellularized materials with or without cells could be analyzed due to the limited number of other combinations. No statistically significant differences were found for the inclusion of cells for any of the outcome measures (p=0.5 for side-effects, p=0.7 for functionality and p=0.08 for study completion).

When comparing pre-clinical and clinical estimated probabilities, point estimates for absence of side-effects after inlay repair seem to be higher in clinical studies for both acellular and cellular templates. For functionality, the point estimates were similar. The estimated probability for study completion was much lower in clinical studies compared to pre-clinical studies regardless of the addition of cells, but these cannot be directly compared due to distinctive definitions for study completion and differences in disease status.

Discussion

Reconstructive surgery using biomaterials has been studied as an alternative approach for urethral repair since the early seventies and efforts along these lines expanded rapidly in the nineties when the term 'Tissue Engineering' was introduced (Fig. 4) [11]. Nowadays, pre-clinical studies have been readily performed, but clinical studies have not followed this trend. Although many (pre-)clinical studies have been performed, tissue engineering is not used as an alternative treatment in routine clinical practice, except for a select patient group with a history of failed repairs [22-24]. In this systematic review, all (pre-)clinical publications on urethra tissue engineering until June 2016 were analyzed to assess the evidence for the efficacy. For clinical studies, the term "effectiveness" may be more suitable, as most studies included in this review showed a heterogeneous patient population [25]. However, we used the term "efficacy" for pre-clinical and clinical studies throughout this systematic review. For both pre-clinical and clinical studies, tissue engineering had a high probability for functionality, defined as voiding with continence. Study completion was high in pre-clinical studies, but not in clinical studies. This may be related to the difference in our definition of study completion and in study design. In pre-clinical studies, animals generally only need to survive for several months to study the tissue regeneration process, compared to patients that need to show a good long term outcome without reintervention and without being lost in follow-up. Most patients had a history of failed repairs using conventional techniques, while healthy animals were used. As randomized clinical studies were lacking, e.g. comparison with gold standard treatments (free skin graft or buccal mucosa urethroplasty) [3], no direct comparisons with current clinical practice could be made. Available literature about complex two-stage urethroplasty shows complication-free rates, functionality and study completion of approximately 62%, 67% and 36% [26], similar to the outcome of tissue engineered urethras (based on point estimates). This suggests that tissue engineered urethras may perform adequately and may be a valid alternative. Clearly, randomized controlled clinical trials are needed to clarify this issue.

Application of cells

There is no consensus on the potential beneficial effects of cell seeding of tissue engineered constructs for the urogenital system. For tissue engineering of the bladder, the addition of cells did not give an overall beneficial effect on tissue regeneration [27], while others claim that cells are required for urethra repair of constructs >0.5 cm [28]. For urethra tissue engineering, the inclusion of cells significantly reduced side effects in pre-clinical studies for both full (p=0.001) and inlay (p=0.003) defects. In other, less comprehensive systematic reviews, a similar outcome regarding the effectiveness of the addition of cells was shown [29, 30]. For full defects, cell addition has more added value, which may be explained by the fact that cells can only infiltrate from the two urethra edges, while in inlay repair cell ingrowth can also occur from the sides, boosting cell coverage.

The effects of cell addition on functionality and study completion were not significant, regardless of surgical procedure. This may be caused by the short follow-up period underestimating long-term complications, such as complete strictures. Meta-analysis of clinical studies showed no significant effect of cells for any of the outcome measures. Consequently, the use of cells for the repair of urethra in the clinic remains debatable.

Type of biomaterial

Meta-analysis showed no differences in estimated probabilities for the different materials in most of the conditions, with the exception of synthetic materials showing better estimated probabilities than natural materials in full circumferential repair without cells regarding side-effects. For inlay repair in pre-clinical studies, synthetic materials did not perform as well as in full repair, but only a limited number of studies was reported.

Decellularized materials were used in the vast majority of clinical studies. This may be related to the experience with decellularized materials in other fields of tissue engineering, such as skin tissue engineering [31]. Which type of biomaterial is superior to the current state-of-the-art remains to be established.

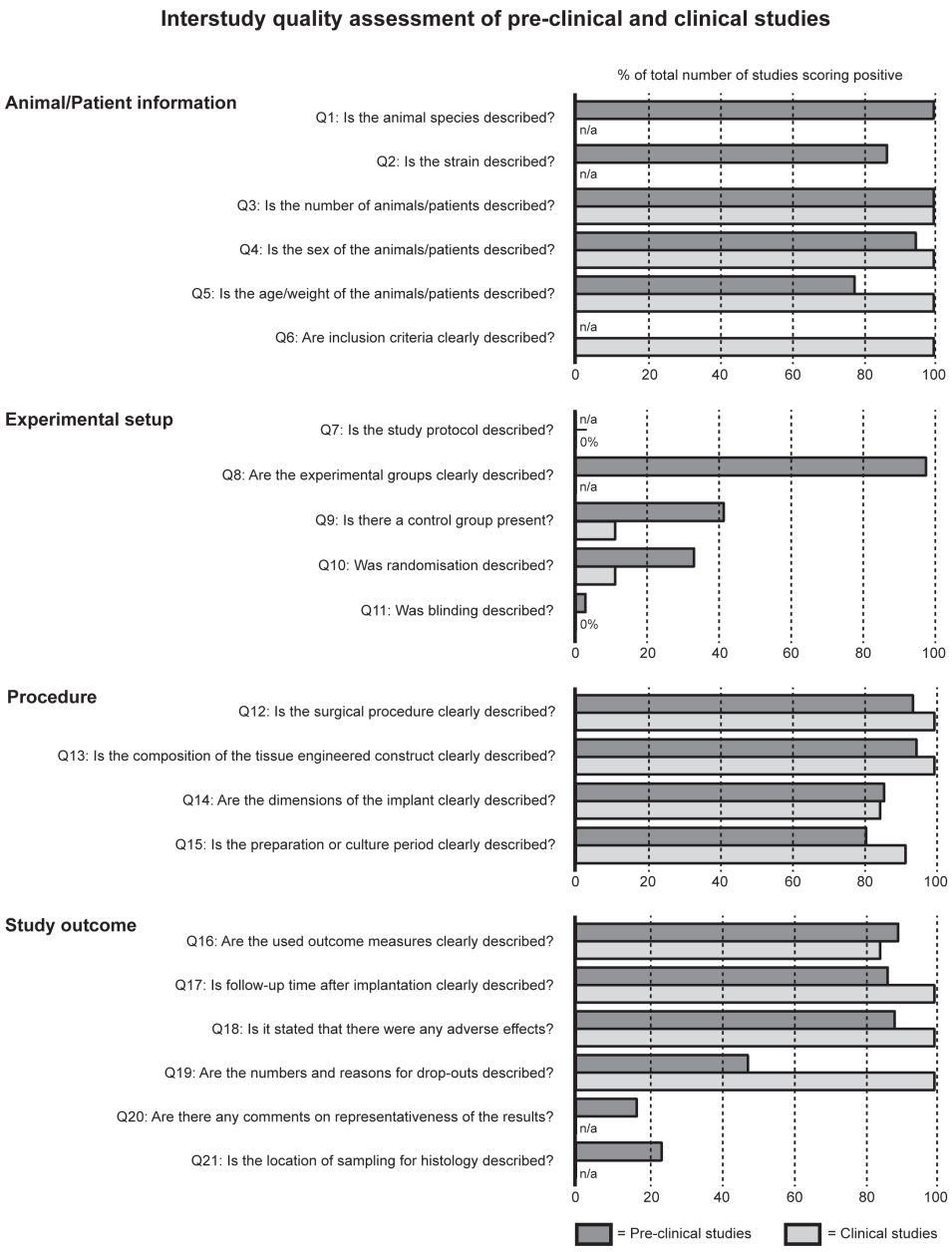

Figure 2. Quality assessment of pre-clinical and clinical studies. All studies included for meta-analysis were scored on clear reporting of several key parameters (Q1-Q21) showing that study design such as inclusion of proper control groups, associated randomization and blinding, reporting of key parameters such as representativeness of shown results and drop-outs needs to be improved in pre-clinical studies.

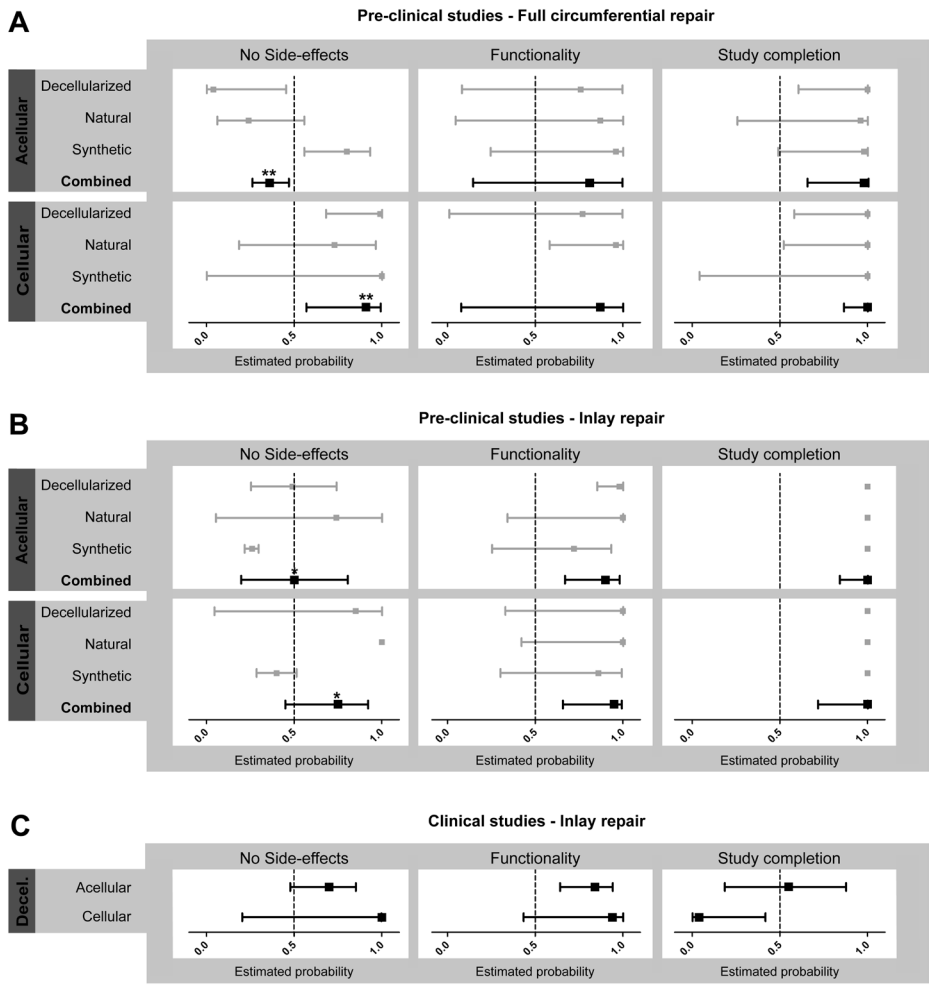

Figure 3. Estimated probability including 95% confidence intervals for the absence of side effects, functionality and study completion for A) full circumferential repair and B) inlay repair in pre-clinical studies, both categorized for the use of cells and the type of biomaterial. C) For the clinical studies, only decellularized material with or without cells could be analyzed. The effect of cells on the three outcome measures was calculated in estimated probabilities. Overall differences for cellular vs. acellular templates were determined for each outcome measure for both full and inlay repair: * p=0.003, ** p=0.001, all other differences were not significant (p>0.05). Specific point estimates and confidence interval are given in Appendix 6.

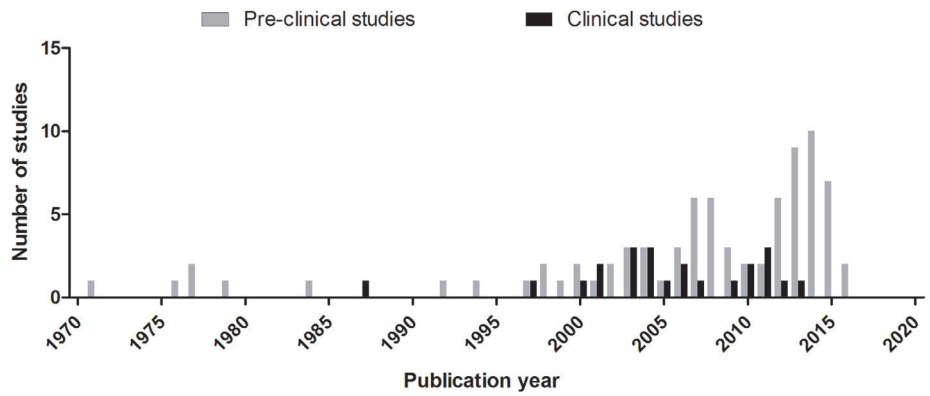

Figure 4. Number of publications per year for pre-clinical and clinical studies included in this systematic review. After several single studies between 1971 and 1994, the number of publications increased. Peaks in both clinical and pre-clinical studies were seen around 2005-2008 and again between 2012-2015.

Selection of animal species

The choice of animal species is often based on financial issues, experience of the researchers, ethical arguments and practical restrictions [32-34]. An evidence-based approach can aid in selection of the most appropriate model. In this review, differences between treatment were not notably influenced by the choice for rabbit or dog, however a higher statistical power would strengthen this claim.

Clinical relevance and limitations of pre-clinical and clinical studies

Quality of the experimental designs and reporting of pre-clinical studies was generally low. Proper control groups, such as sham operation groups and gold standard treatment groups, were often lacking. Instead, the experimental material without cells was generally considered the control. In addition, outcome measures and drop-outs were not specifically reported for each animal, complicating data interpretation. Also, representativeness of presented data was often not mentioned. This may have hampered clinical translation of these pre-clinical findings. To improve this, all design parameters and outcomes should be specifically documented for individual animals similar to patients in clinical studies. The "Gold standard publication checklist to improve the quality of animal studies" by Hooijmans et al. would be helpful for the design and reporting of pre-clinical studies [35].

Another limitation for the level of evidence provided by the pre-clinical studies is the use of healthy animals, in which a created defect is immediately closed, compared to patients with a history of stricture, lichen sclerosis or hypospadias. From the patients in clinical studies 75% had one or more previous treatments, e.g. dilation, urethrotomy or urethroplasty, before attempting the tissue engineered constructs. The requirement of animal models with injury or disease has been shown in other fields [36] and should also be considered in tissue engineering, in this particular situation by inducing strictures.

Clinical studies provided a low level of evidence due to their setup, making the true effect of tissue engineering as surrogate for the current standard treatment unclear. Only El-Kassaby et al. (Table 2, #3) performed a small randomized controlled study. To improve the level of evidence, more randomized controlled studies are needed, preferably with larger numbers of patients and longer follow-up. Compared to the pre-clinical studies, reporting of important parameters was much better, notably regarding drop-outs and adverse events. Nevertheless, to further improve the quality of the clinical studies, the study protocol should be published with the manuscript and a detailed description of patient inclusion criteria (e.g. sex, age and medical history) should be provided.

The level of evidence is further limited by original research's susceptibility to publication bias [37], which may lead to overestimation of the treatment effect in pre-clinical studies. Recognition of this bias may partly explain the poor translation of tissue engineering techniques to the clinic.

Furthermore, pre-clinical studies should better support the clinical need: the majority of pre-clinical studies involves full circumferential repair, where clinicians mainly perform inlay repair [3]. This may be explained by pre-clinical researchers attempting to prove the effectiveness of the experimental treatment for the most problematic (circumferential) procedures, assuming that it will also be effective in less complicated (inlay) approaches.

Finally, inclusion of cells remains challenging in a clinical setting as no beneficial effect was seen (in 11 patients), even though this significantly improved pre-clinical outcome. It is possible that inclusion of cells was perceived as too problematic, despite better results in a pre-clinical setting and that in the final assessment the choice was driven by parameters other than pre-clinical outcome. To consider cells for clinical applications, its efficacy has to be proven as the use of cells involves extensive regulatory requirements which may hamper clinical application [38-40]. In addition, the costs of cellular implants will be higher compared to off-the-shelf acellular implants, since two procedures are needed (cell harvesting in urine or biopsy, and urethroplasty) and in vitro cell expansion may be needed [41, 42].

Conclusions

The efficacy of tissue engineering for urethra repair could not be determined due to a lack of controlled (pre-)clinical studies. However, meta-analysis outcomes (side-effects, functionality and study completion) were comparable to current treatment options described in literature, indicating the potential of tissue engineering for urethra repair. The findings of this systematic review may result in improved study design which may aid the translation of tissue engineered urethras to the clinic as an alternative for autografts.

Acknowledgements

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Appendix 1

Search strategy to retrieve all relevant literature using PubMed. Individual search components for tissue engineering, urethra, pre-clinical- and clinical studies were designed using relevant MeSH terms and other relevant synonyms and related terms. Search components 1, 2 and 3A were combined to retrieve all relevant pre-clinical studies, while search components 1,2 and 3B were combined to retrieve all clinical studies.

Search component 1: Tissue Engineering

tissue engineering [MeSH] OR tissue culture techniques [MeSH] OR organ culture techniques [MeSH] OR organoids [MeSH] OR guided tissue regeneration [MeSH] OR regenerative medicine [MeSH] OR artificial organs [MeSH] OR tissue scaffolds [MeSH] OR biocompatible materials [MeSH] OR bioreactors [MeSH] OR (regenerative [tiab] AND (medicine [tiab] OR medicines [tiab])) OR ((decellularized [tiab] OR acellular [tiab] OR cell-free [tiab] bioartificial [tiab] OR bio-artificial [tiab] OR artificial [tiab] OR tissue [tiab] OR tissues [tiab] OR organ [tiab] OR organs [tiab] OR culture [tiab] OR cultures [tiab]) AND (autograft [tiab] OR autografts [tiab] OR graft [tiab] OR grafts [tiab] OR matrix [tiab] OR matrices [tiab] OR biomatrix [tiab] OR biomatrices [tiab] or biomaterial [tiab] OR biomaterials [tiab] OR scaffold [tiab] OR scaffolds [tiab] OR scaffolding [tiab] OR engineering [tiab] OR engineer [tiab] OR culture [tiab] OR cultures [tiab] OR regeneration [tiab] OR regenerated [tiab] OR regenerating [tiab] OR reconstruction [tiab] OR reconstructed [tiab] OR reconstructing [tiab])) OR tissue-engineered [tiab] OR tissue engineering [tiab] OR bio-engineering [tiab] OR bioengineering [tiab] OR bioengineered [tiab] OR bio-engineered [tiab] OR organoids [tiab] OR organoid [tiab] OR bioartificial [tiab] OR bio-artificial [tiab] OR artificial [tiab] OR scaffold [tiab] OR scaffolds [tiab] OR scaffolding [tiab] OR matrix [tiab] OR matrices [tiab] OR biomatrix [tiab] OR biomatrices [tiab] OR biomaterial [tiab] OR biomaterials [tiab] OR bioreactor [tiab] OR bioreactors [tiab]

Search component 2: Urethra

urethra [MeSH] OR urethral diseases [MeSH] OR urethra [tiab] OR urethral [tiab] OR urethras [tiab] OR urethrotomy [tiab] OR urethrotomies OR ureterotomy [tiab] OR ureterotomies [tiab] OR urethrotomia [tiab] OR urethroplasty OR urethroplasties [tiab] OR ((stricture [tiab] OR strictures [tiab]) AND (urology [tiab] OR urinary [tiab] OR urine [tiab] OR urological [tiab])) OR hypospadias [tiab] OR epispadias [tiab] OR urethritis [tiab] OR (meatus [tiab] AND (urology [tiab] OR urinary [tiab] OR urine [tiab] OR urological [tiab])) OR chordee [tiab] OR (perineal [tiab] AND (urology [tiab] OR urinary [tiab] OR urine [tiab] OR urological [tiab]))

Search component 3A: preclinical studies

("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSH Terms:noexp] OR

"chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSH Terms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSH Terms:noexp] OR "primates"[MeSH Terms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSH Terms:noexp] OR "strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR "catarrhini"[MeSH Terms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "hominidae"[MeSH Terms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongo pygmaeus"[MeSH Terms]) OR ((animals[tiab] OR animal[tiab] OR mice[Tiab] OR mus[Tiab] OR mouse[Tiab] OR murine[Tiab] OR woodmouse[tiab] OR rats[Tiab] OR rat[Tiab] OR murinae[Tiab] OR muridae[Tiab] OR cottonrat[tiab] OR cottonrats[tiab] OR hamster[tiab] OR hamsters[tiab] OR cricetinae[tiab] OR rodentia[Tiab] OR rodent[Tiab] OR rodents[Tiab] OR pigs[Tiab] OR pig[Tiab] OR swine[tiab] OR swines[tiab] OR piglets[tiab] OR piglet[tiab] OR boar[tiab] OR boars[tiab] OR "sus scrofa"[tiab] OR ferrets[tiab] OR ferret[tiab] OR polecat[tiab] OR polecats[tiab] OR "mustela putorius"[tiab] OR "guinea pigs"[Tiab] OR "guinea pig"[Tiab] OR cavia[Tiab] OR callithrix[Tiab] OR marmoset[Tiab] OR marmosets[Tiab] OR cebuella[Tiab] OR hapale[Tiab] OR octodon[Tiab] OR chinchilla[Tiab] OR chinchillas[Tiab] OR gerbillinae[Tiab] OR gerbil[Tiab] OR gerbils[Tiab] OR jird[Tiab] OR jirds[Tiab] OR merione[Tiab] OR meriones[Tiab] OR rabbits[Tiab] OR rabbit[Tiab] OR hares[Tiab] OR hare[Tiab] OR diptera[Tiab] OR 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baby"[Tiab] OR bush babies[Tiab] OR galagos[Tiab] OR galago[Tiab] OR pongidae[Tiab] OR gorilla[Tiab] OR gorillas[Tiab] OR "pongo pygmaeus"[Tiab] OR orangutan[Tiab] OR orangutans[Tiab] OR lemur[Tiab] OR lemurs[Tiab] OR lemuridae[Tiab] OR horse[Tiab] OR horses[Tiab] OR equus[Tiab] OR cow[Tiab] OR calf[Tiab] OR bull[Tiab] OR chicken[Tiab] OR chickens[Tiab] OR gallus[Tiab] OR quail[Tiab] OR bird[Tiab] OR birds[Tiab] OR quails[Tiab] OR poultry[Tiab] OR poultries[Tiab] OR fowl[Tiab] OR fowls[Tiab] OR reptile[Tiab] OR reptilia[Tiab] OR reptiles[Tiab] OR snakes[Tiab] OR snake[Tiab] OR lizard[Tiab] OR lizards[Tiab] OR alligator[Tiab] OR alligators[Tiab] OR crocodile[Tiab] OR crocodiles[Tiab] OR turtle[Tiab] OR turtles[Tiab] OR amphibian[Tiab] OR amphibians[Tiab] OR amphibia[Tiab] OR frog[Tiab] OR frogs[Tiab] OR bombina[Tiab] OR salientia[Tiab] OR toad[Tiab] OR toads[Tiab] OR "epidalea calamita"[Tiab] OR salamander[Tiab] OR salamanders[Tiab] OR eel[Tiab] OR eels[Tiab] OR fish[Tiab] OR 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parakeets[Tiab] OR parrot[Tiab] OR parrots[Tiab] OR donkey[Tiab] OR donkeys[Tiab] OR mule[Tiab] OR mules[Tiab] OR zebra[Tiab] OR zebras[Tiab] OR shrew[Tiab] OR shrews[Tiab] OR bison[Tiab] OR bisons[Tiab] OR buffalo[Tiab] OR buffaloes[Tiab] OR deer[Tiab] OR deers[Tiab] OR bear[Tiab] OR bears[Tiab] OR panda[Tiab] OR pandas[Tiab] OR "wild hog"[Tiab] OR "wild boar"[Tiab] OR fitchew[Tiab] OR fitch[Tiab] OR beaver[Tiab] OR beavers[Tiab] OR jerboa[Tiab] OR jerboas[Tiab] OR capybara[Tiab] OR capybaras[Tiab]) NOT medline[sb])

Search component 3B: clinical studies

(((clinical[Title/Abstract] AND trial[Title/Abstract]) OR clinical trials[MeSH Terms] OR clinical trial[Publication Type] OR random*[Title/Abstract] OR random allocation[MeSH Terms] OR therapeutic use[MeSH Subheading])) OR ((Human[tiab] OR humans[tiab] OR patient[tiab] OR patients[tiab]) AND (study[tiab] OR studies[tiab] OR research[tiab] OR investigat*[tiab] OR clinic*[tiab] OR therapy[tiab] OR treatment[tiab] OR surgery[tiab] OR surgical[tiab]))

Appendix 2

Search strategy to retrieve all relevant literature using Embase. Individual search components for tissue engineering, urethra, pre-clinical- and clinical studies were designed using relevant EMTree-terms and relevant synonyms and related terms. Search components 1, 2 and 3A were combined to retrieve all relevant pre-clinical studies, while search components 1,2 and 3B were combined to retrieve all clinical studies.

Search component 1: Tissue Engineering

Exp tissue engineering/ OR Exp tissue culture/ OR Exp organ culture/ OR Exp tissue regeneration/ OR Exp regenerative medicine/ OR Exp artificial organ/ OR Exp tissue scaffold/ OR Exp biomaterial/ OR Exp bioreactor/ OR (regenerative AND (medicine OR medicines)).ti,ab. OR ((decellularized OR acellular OR cell-free bioartificial OR bio-artificial OR artificial OR tissue OR tissues OR organ OR organs OR culture OR cultures) AND (autograft OR autografts OR graft OR grafts OR matrix OR matrices OR biomatrix OR biomatrices OR biomaterial OR biomaterials OR scaffold OR scaffolds OR scaffolding OR engineering OR engineer OR culture OR cultures OR regeneration OR regenerated OR regenerating OR reconstruction OR reconstructed OR reconstructing)).ti,ab. OR (tissue-engineered OR tissue engineering OR bio-engineering OR bioengineering OR bioengineered OR bio-engineered OR organoids OR organoid OR bioartificial OR bio-artificial OR artificial OR scaffold OR scaffolds OR scaffolding OR matrix OR matrices OR biomatrix OR biomatrices OR biomaterial OR biomaterials OR bioreactor OR bioreactors).ti,ab.

Search component 2: Urethra

Exp urethra/ OR Exp urethra disease/ OR Exp urethra surgery/ OR urethra.ti,ab. OR urethral.ti,ab. OR urethras.ti,ab. OR urethrotomy.ti,ab. OR urethrotomies.ti,ab. OR ureterotomy.ti,ab. OR ureterotomies.ti,ab. OR urethrotomia.ti,ab. OR urethroplasty.ti,ab. OR urethroplasties.ti,ab. OR ((stricture OR strictures) AND (urology OR urinary OR urine OR urological)).ti,ab. OR hypospadias.ti,ab. OR epispadias.ti,ab. OR urethritis.ti,ab. OR (meatus AND (urology OR urinary OR urine OR urological)).ti,ab. OR chordee.ti,ab. OR (perineal AND (urology OR urinary OR urine OR urological)).ti,ab.

Search component 3A: preclinical studies

exp animal experiment/ or exp animal model/ or exp experimental animal/ or exp transgenic animal/ or exp male animal/ or exp female animal/ or exp juvenile animal/ OR animal/ OR chordata/ OR vertebrate/ OR tetrapod/ OR exp fish/ OR amniote/ OR exp amphibia/ OR mammal/ OR exp reptile/ OR exp sauropsid/ OR therian/OR exp monotremate/ OR placental mammals/ OR exp marsupial/ OR Euarchontoglires/ OR exp Afrotheria/ OR exp Boreoeutheria/ OR exp Laurasiatheria/ OR exp Xenarthra/ OR primate/ OR exp Dermoptera/ OR exp Glires/ OR exp Scandentia/ OR Haplorhini/ OR exp prosimian/ OR simian/ OR exp tarsiiform/ OR Catarrhini/ OR exp Platyrrhini/ OR ape/ OR exp

Cercopithecidae/ OR hominid/ OR exp hylobatidae/ OR exp chimpanzee/ OR exp gorilla/ OR exp orang utan/ OR (animal OR animals OR pisces OR fish OR fishes OR catfish OR catfishes OR sheatfish OR silurus OR arius OR heteropneustes OR clarias OR gariepinus OR fathead minnow OR fathead minnows OR pimephales OR promelas OR cichlidae OR trout OR trouts OR char OR chars OR salvelinus OR salmo OR oncorhynchus OR guppy OR guppies OR millionfish OR poecilia OR goldfish OR goldfishes OR carassius OR auratus OR mullet OR mullets OR mugil OR curema OR shark OR sharks OR cod OR cods OR gadus OR morhua OR carp OR carps OR cyprinus OR carpio OR killifish OR eel OR eels OR anguilla OR zander OR sander OR lucioperca OR stizostedion OR turbot OR turbots OR psetta OR flatfish OR flatfishes OR plaice OR pleuronectes OR platessa OR tilapia OR tilapias OR oreochromis OR sarotherodon OR common sole OR dover sole OR solea OR zebrafish OR zebrafishes OR danio OR rerio OR seabass OR dicentrarchus OR labrax OR morone OR lamprey OR lampreys OR petromyzon OR pumpkinseed OR pumpkinseeds OR lepomis OR gibbosus OR herring OR clupea OR harengus OR amphibia OR amphibian OR amphibians OR anura OR salientia OR frog OR frogs OR rana OR toad OR toads OR bufo OR xenopus OR laevis OR bombina OR epidalea OR calamita OR salamander OR salamanders OR newt OR newts OR triturus OR reptilia OR reptile OR reptiles OR bearded dragon OR pogona OR vitticeps OR iguana OR iguanas OR lizard OR lizards OR anguis fragilis OR turtle OR turtles OR snakes OR snake OR aves OR bird OR birds OR quail OR quails OR coturnix OR bobwhite OR colinus OR virginianus OR poultry OR poultries OR fowl OR fowls OR chicken OR chickens OR gallus OR zebra finch OR taeniopygia OR guttata OR canary OR canaries OR serinus OR canaria OR parakeet OR parakeets OR grasskeet OR parrot OR parrots OR psittacine OR psittacines OR shelduck OR tadorna OR goose OR geese OR branta OR leucopsis OR woodlark OR lullula OR flycatcher OR ficedula OR hypoleuca OR dove OR doves OR geopelia OR cuneata OR duck OR ducks OR greylag OR graylag OR anser OR harrier OR circus pygargus OR red knot OR great knot OR calidris OR canutus OR godwit OR limosa OR lapponica OR meleagris OR gallopavo OR jackdaw OR corvus OR monedula OR ruff OR philomachus OR pugnax OR lapwing OR peewit OR plover OR vanellus OR swan OR cygnus OR columbianus OR bewickii OR gull OR chroicocephalus OR ridibundus OR albifrons OR great tit OR parus OR aythya OR fuligula OR streptopelia OR risoria OR spoonbill OR platalea OR leucorodia OR blackbird OR turdus OR merula OR blue tit OR cyanistes OR pigeon OR pigeons OR columba OR pintail OR anas OR starling OR sturnus OR owl OR athene noctua OR pochard OR ferina OR cockatiel OR nymphicus OR hollandicus OR skylark OR alauda OR tern OR sterna OR teal OR crecca OR oystercatcher OR haematopus OR ostralegus OR shrew OR shrews OR sorex OR araneus OR crocidura OR russula OR european mole OR talpa OR chiroptera OR bat OR bats OR eptesicus OR serotinus OR myotis OR dasycneme OR daubentonii OR pipistrelle OR pipistrellus OR cat OR cats OR felis OR catus OR feline OR dog OR dogs OR canis OR canine OR canines OR otter OR otters OR lutra OR badger OR badgers OR meles OR fitchew OR fitch OR foumart or foulmart OR ferrets OR ferret OR polecat OR polecats OR mustela OR putorius OR weasel OR weasels OR fox OR foxes OR vulpes OR common seal OR phoca OR vitulina OR grey seal OR halichoerus OR horse OR horses OR equus OR equine OR equidae OR donkey OR donkeys OR mule OR mules OR pig OR pigs OR swine OR swines OR hog OR hogs OR boar OR boars OR porcine OR piglet OR piglets OR sus OR scrofa OR llama OR llamas OR lama OR glama OR deer OR deers OR cervus OR elaphus OR cow OR cows OR bos taurus OR bos indicus OR bovine OR bull OR bulls OR cattle OR bison OR bisons OR sheep OR sheeps OR ovis aries OR ovine OR lamb OR lambs OR mouflon OR mouflons OR goat OR goats OR capra OR caprine OR chamois OR rupicapra OR leporidae OR lagomorpha OR lagomorph OR rabbit OR rabbits OR oryctolagus OR cuniculus OR laprine OR hares OR lepus OR rodentia OR rodent OR rodents OR murinae OR mouse OR mice OR mus OR musculus OR murine OR woodmouse OR apodemus OR rat OR rats OR rattus OR norvegicus OR guinea pig OR guinea pigs OR cavia OR porcellus OR hamster OR hamsters OR mesocricetus OR cricetulus OR cricetus OR gerbil OR gerbils OR jird OR jirds OR meriones OR unguiculatus OR jerboa OR jerboas OR jaculus OR chinchilla OR chinchillas OR beaver OR beavers OR castor fiber OR castor canadensis OR sciuridae OR squirrel OR squirrels OR sciurus OR chipmunk OR chipmunks OR marmot OR marmots OR marmota OR suslik OR susliks OR spermophilus OR cynomys OR cottonrat OR cottonrats OR sigmodon OR vole OR voles OR microtus OR myodes OR glareolus OR primate OR primates OR prosimian OR prosimians OR lemur OR lemurs OR lemuridae OR loris OR bush baby OR bush babies OR bushbaby OR bushbabies OR galago OR galagos OR anthropoidea OR anthropoids OR simian OR simians OR monkey OR monkeys OR marmoset OR marmosets OR callithrix OR cebuella OR tamarin OR tamarins OR saguinus OR leontopithecus OR squirrel monkey OR squirrel monkeys OR saimiri OR night monkey OR night monkeys OR owl monkey OR owl monkeys OR douroucoulis OR aotus OR spider monkey OR spider monkeys OR ateles OR baboon OR baboons OR papio OR rhesus monkey OR macaque OR macaca OR mulatta OR cynomolgus OR fascicularis OR green monkey OR green monkeys OR chlorocebus OR vervet OR vervets OR pygerythrus OR hominoidea OR ape OR apes OR hylobatidae OR gibbon OR gibbons OR siamang OR siamangs OR nomascus OR symphalangus OR hominidae OR orangutan OR orangutans OR pongo OR chimpanzee OR chimpanzees OR pan troglodytes OR bonobo OR bonobos OR pan paniscus OR gorilla OR gorillas OR troglodytes).ti,ab.

Search component 3B: clinical studies

Exp clinical trial/ OR (clinical AND (trial OR trials)).ti,ab. OR Exp randomization/ OR Exp therapy/ OR Exp treatment outcome/ OR Exp controlled study/ OR exp health care quality/ OR random:.tw. or clinical trial:.mp.

Appendix 3

Reference list on alphabetical order of preclinical studies included in Table 1.

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Appendix 4

Reference list on alphabetical order of clinical studies included in Table 2.

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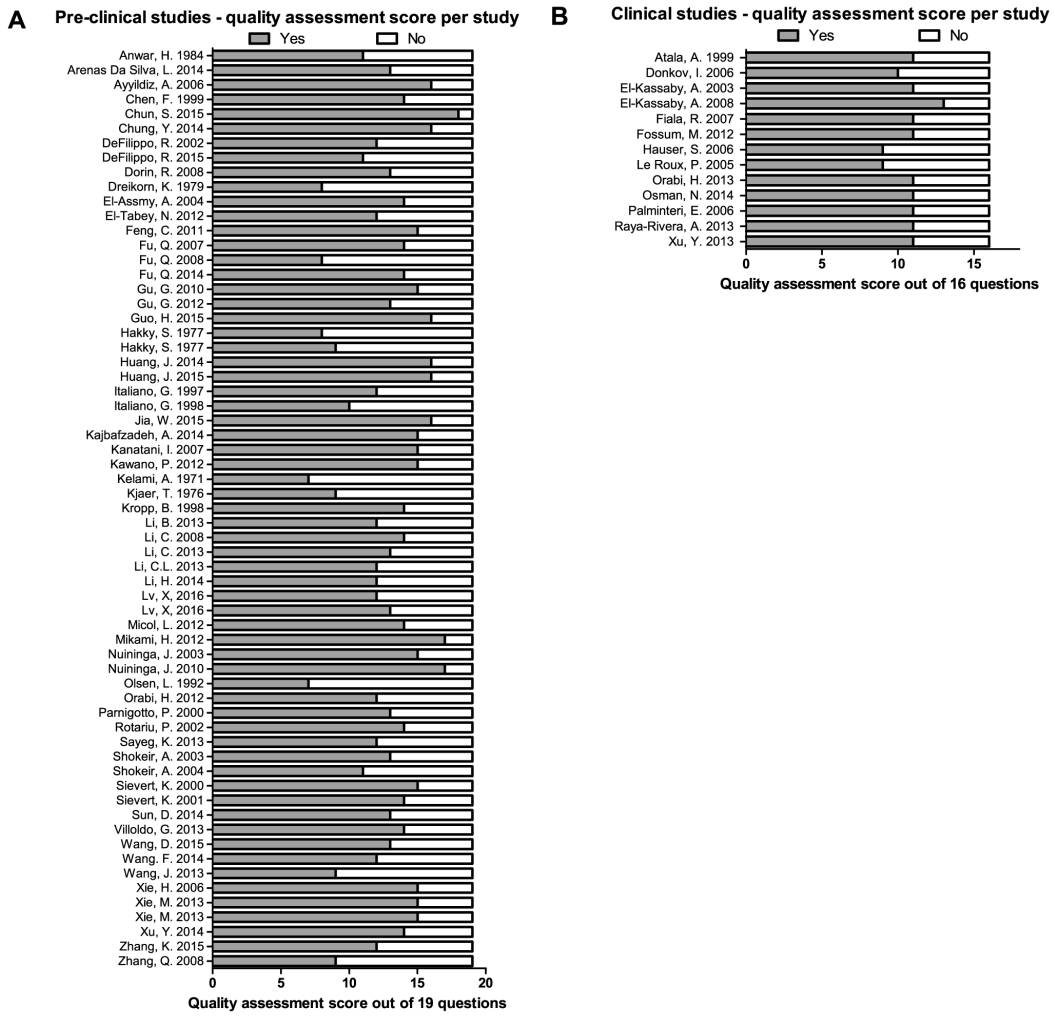
Appendix 5

Quality assessment score per study for (A) pre-clinical studies and (B) clinical studies. Quality was scored based on 19 questions for pre-clinical studies and 17 questions for clinical studies (see Fig. 2). Detailed scores per study are available upon request.



Appendix 6

Numerical data on which Figure 3 is based. Estimated probability including 95% confidence intervals for the absence of side effects, functionality and study completion for A) full circumferential repair and B) inlay repair in pre-clinical studies, both categorized for the use of cells and the type of biomaterial. C) For the clinical studies, only decellularized material with or without cells could be analyzed. The effect of cells on the three outcome measures was calculated in estimated probabilities. Overall differences for cellular vs. acellular templates were determined for each outcome measure for both full and inlay repair:
* p=0.003, ** p=0.001, all other differences were not significant (p>0.05).

A. Pre-clinical studies – Full circumferential repair

		Point estimate and 95% CI [lower:upper]		
	Biomaterial type	No side-effects	Functionality	Study completion
Acellular	Decellularized	0.04 [0.001:0.46]	0.76 [0.09:0.99]	1.0 [0.63:1.0]
	Natural	0.24 [0.07:0.57]	0.87 [0.04:1.0]	0.95 [0.26:1.0]
	Synthetic	0.80 [0.56:0.93]	0.96 [0.25:1.0]	0.98 [0.49:1.0]
	Overall	**0.36 [0.26:0.48]**	**0.81 [0.14:0.99]**	**0.98 [0.66:1:0]**
Cellular	Decellularized	0.99 [0.68:1.0]	0.77 [0.01:0.99]	1.0 [0.58:1.0]
	Natural	0.73 [0.19:0.97]	1.0 [0.59:1.0]	1.0 [0.52:1.0]
	Synthetic	1.0 [0.00:1.0]	unable to estimate	1.0 [0.03:1.0]
	Overall	**0.91 [0.59:0.99]**	**0.87 [0.07:1.0]**	**1:0 [0.88:1.0]**

B. Pre-clinical studies – Inlay repair

		Point estimate and 95% CI [lower:upper]		
	Biomaterial type	No side-effects	Functionality	Study completion
Acellular	Decellularized	0.49 [0.26:0.73]	0.98 [0.88:1.0]	unable to estimate
	Natural	0.74 [0.04:1.0]	1.0 [0.36:1.0]	unable to estimate
	Synthetic	0.26 [0.22:0.29]	0.73 [0.26:0.95]	unable to estimate
	Overall	**0.50 [0.20:0.80]**	**0.90 [0.66:0.98]**	**1.0 [0.92:1.0]**
Cellular	Decellularized	0.85 [0.03:1.0]	1.0 [0.34:1.0]	unable to estimate
	Natural	1.0 [1.0:1.0]	1.0 [0.42:1.0]	unable to estimate
	Synthetic	0.40 [0.29:0.52]	0.86 [0.31:0.99]	unable to estimate
	Overall	**0.75 [0.44:0.92]**	**0.95 [0.66:0.99]**	**1:0 [0.75:1.0]**

C. Clinical studies – Inlay repair

		Point estimate and 95% CI [lower:upper]		
	Biomaterial type	No side-effects	Functionality	Study completion
De-cel.	Acellular	0.70 [0.49:0.85]	0.84 [0.64:0.94]	0.55 [0.18:0.87]
	Cellular	1.0 [0.20:1.0]	0.94 [0.43:1.0]	0.04 [0.002:0.43]

Part 2

New hybrid biomaterials in urogenital tissue engineering

Chapter 5

The effect of a cyclic uniaxial strain on urinary bladder cells

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Abstract

Purpose: Pre-conditioning of a cell seeded construct may improve the functional outcome of a tissue engineered construct for augmentation cystoplasty. The precise effects of mechanical stimulation on urinary bladder cells in vitro is not clear. In this study we investigate the effect of a cyclic uniaxial strain culture on urinary bladder cells which were seeded on a type I collagen scaffold.

Methods: Isolated porcine smooth muscle cells or urothelial cells were seeded on a type I collagen scaffolds and cultured under static and dynamic conditions. A uniform cyclic uniaxial strain was applied to the seeded scaffold using a Bose Electroforce Bio-Dynamic bioreactor. Cell proliferation rate and phenotype were investigated, including SEM analysis, RT-PCR and immunohistochemistry for α-Smooth muscle actin, Calponin-1, desmin and RCK103 expression to determine the effects of mechanical stimulation on both cell types.

Results: Dynamic stimulation of smooth muscle cell seeded constructs resulted in cell alignment and enhanced proliferation rate. Additionally, expression of α-Smooth muscle actin and calponin-1 was increased suggesting differentiation of smooth muscle cells to a more mature phenotype.

Conclusions: Mechanical stimuli did not enhance the proliferation and differentiation of urothelial cells. Mechanical stimulation i.e., preconditioning may improve the functional in vivo outcome of smooth muscle cell seeded constructs for flexible organs such as the bladder.

Introduction

For patients who need bladder reconstruction, a tissue engineered bladder may be an alternative to current procedures in which autologous bowel tissue is used. The first clinical studies with cell seeded bladder-sized constructs illustrated that scaffolds implanted in patients who had a normal bladder cycle regenerated properly, while patients with abnormal cycles responded poorly [1]. Bladder regeneration studies in animals indicated slow smooth muscle cell ingrowth in scaffolds, probably due to the limited migration from adjacent tissue [2]. This suggests that adequate conditioning of the tissue engineered construct may be needed to improve the functional outcome of the regenerated tissue for flexible organs.

It has become clear that mechanical stimulation is equally important in cellular behavior as genetic and chemical signals [3]. By providing mechanotransduction, cell proliferation and differentiation can be influenced and it may lead to extracellular matrix (ECM) production [4-6]. Therefore, it is important to investigate the behavior and phenotype of cells in constructs while under defined mechanical strain before implantation.

A bioreactor can apply mechanical stimuli under controlled in vitro conditions [7, 8]. Initially vacuum suction (Seliktar et al., 2000) and mechanical stretch was used [5]. Thereafter different bioreactor systems using hydrostatic pressures have been developed and used to study urinary bladder tissue engineering [9]. Hydrostatic pressure on human bladder smooth muscle cells on aligned nano fibrous scaffolds resulted in functional improvement of the engineered tissue [10]. Although this setting simulated in vivo conditions, the exact impact of the mechanical stimulation on the urinary bladder cells is not clear. Moreover, whether mechanical stimulation of cells seeded on other materials such as collagen also leads to functional improvement is currently unclear. In this study we investigated the effect of a long-term controlled cyclic uniaxial strain on urinary bladder cells which were seeded on a type I collagen scaffold mimicking the filling and emptying of the bladder to assess whether this pre-conditioning step is beneficial in urinary bladder tissue engineering.

Material and Methods

Products were purchased from Life technologies (Carlsbad; US) unless otherwise indicated.

Scaffolds preparation and characterization

For the construction of collagen scaffolds, insoluble type I collagen fibrils were purified from pulverized bovine Achilles tendon, as previously described [11]. In short, a 0.5% (w/v) type I collagen suspension was made by swelling and subsequent homogenization in 0.25 M acetic acid at 4°C. The collagen suspension was deaerated, poured into six-well plates, frozen at -20 °C and lyophilized. The dried collagen matrices were stabilized using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (Fluka, Sigma-Aldrich; St Louis, US) and N-hydroxysuccinimide (Fluka, Sigma-Aldrich; St Louis, US) crosslinking in 50 mM 2-morpholinoethane

sulphonic acid (MES, pH5,0) containing 40% (v/v) ethanol for 4 h at 21°C. After cross-linking, the scaffolds were washed consecutively in 0.1M Na2HPO4, 1M NaCl, 2M NaCl, demineralized water, disinfected by 70% ethanol washings and stored at -20°C. The degree of crosslinking of the scaffolds was determined by 2,4,6-trinitrobenzene sulfonic acid (TNBS) analysis in triplicate [12]. Collagen strips were cut to match the bioreactor dimensions (length 2.5 cm, width 1 cm), and washed in 70% ethanol (at least 2 times 1 h and 1 time overnight (o/n)), followed by washings in sterile phosphate-buffered saline (PBS; pH 7.4, at least 2 times 1 h and 1 time o/n), and an o/n incubation in medium.

Cell isolation

Porcine bladders were collected from a local abattoir. After opening the bladder, ~1 cm2 pieces were collected under aseptic conditions and tissue specimens were transferred to transport medium (HBSS+Mg+Ca, 10 mM HEPES; pH 7.6, aprotinin 1 µg/ml (Roche; Basel, Switzerland), 100 U/ml penicillin and 100 µg/ml streptomycin (P/S). Tissue specimens were incubated 16 h at 4°C in stripper medium (HBSS-Mg-Ca, 10 mM HEPES; pH 7.6, aprotinin 1 ug/ml (Roche; Basel, Switzerland), P/S and 2.4 U/ml dispase II (Sigma-Aldrich; St Louis, US). Urothelial cells (UC) were isolated by scraping the urothelial sheet using tweezers. Urothelial sheets were collected in a 15 ml tube and incubated for 20 min at 37°C with 100 U/ml collagenase type IV (Sigma-Aldrich; St Louis, US) prepared in transport medium. Cells were collected by centrifugation at 400g for 8min. and seeded in Primaria flasks (BD Falcon®, US; 1 T75 per cm2 tissue specimen). Cells were cultured in keratinocyte serum-free medium (KSFM) supplemented with 50 µg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, 30 ng/ml cholera toxin (Sigma-Aldrich; St Louis, US) and P/S (UC medium).

For the isolation of SMC, the remaining tissue was cut into small pieces (~2 mm2) and incubated for 1.5 h at 37°C with 1.5 U/ml liberase enzyme (Roche; Basel, Switzerland) diluted in HBSS+Ca+Mg and P/S. After vigorous resuspension, the material was pushed through a 70 µm cell strainer (BD Falcon®, US) to remove undigested particles. Cells were collected by centrifugation and cultured in smooth muscle cell medium (SMCM, Sciencell; Carlsbad, US), supplemented with 2% (v/v) fetal bovine serum, 1% (v/v) smooth muscle growth supplement and P/S (2 T75 per cm2 tissue specimen). Cultures were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO2 in air. Medium was changed three times a week and cells were split when 100% confluence was reached. Cells harvested from one porcine bladder were used to prevent the influence of individual differences between animals.

Cells from passage 1 to 3 were used.

Bioreactor culture

Collagen scaffold strips were placed in a 6 well plate and seeded statically with 1 to 1.5 x106 SMC or UC in a volume of 100 µl of SMC medium or UC medium. After 1 hour the volume was increased to 2.5 ml SMCM medium or UC medium. One day after seeding, scaffolds were placed in a Bose Electroforce Bio-Dynamic bioreactor (Fig. 1a). Subsequently the bioreactor chamber was filled with 200 ml RPMI supplemented with 10% FCS, 2mM glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin and cultured under dynamic conditions. A cyclic uniaxial strain was applied with a continuous 0.3 µm/sec cycle strain (20% full stretch followed by folding in 4 h) (Fig. 1b). Control scaffolds were cultured under static conditions in a T75 flask. After 6 days of culture, scaffolds were harvested and processed for evaluation.

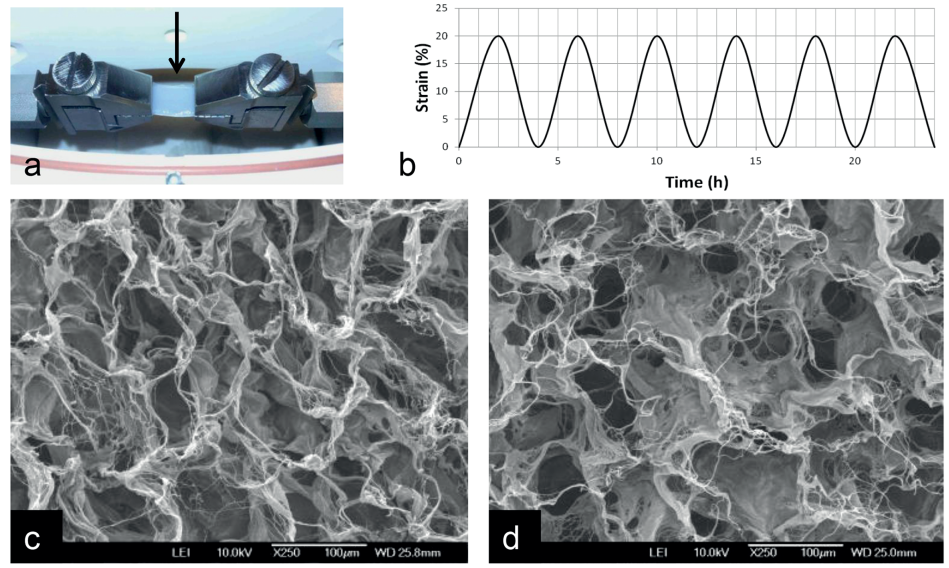

Figure 1. *Overview of the experimental setting with a strip of scaffold clamped in the Bose Electroforce Bio-Dynamic bioreactor (arrow) (a). Overview bioreactor program with a continuous 0.3 µm/sec cycle strain (20% full stretch and fold within 4 h) (b). Scanning electron microscopic pictures of the used type I collagen scaffold (250x); c and d represents the air and pan side, respectively, showing a typical honeycomb structure.*

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on isolated cells (in triplicate) and cells cultured on scaffolds (once for every static/dynamic sample). Materials were washed three times for 1 h with PBS and fixed for at least 1 h in 2% (v/v) glutaraldehyde (Merck, Kenilworth, New Jersey, US) in 0.1 M phosphate buffer (PB), pH 7.4 at 21°C. Scaffolds were washed three times (1 h) using 0.1 M PB. For dehydration scaffolds were washed for at least 1 h in increasing concentrations of ethanol (30%, 50%, 70%, 100% (v/v)) and one additional o/n washing step in 100% ethanol. Scaffolds were critical point dried (CPD) using liquid CO2. After drying, gold sputtering was performed prior to SEM analysis (JEOL JSM-6310; Tokyo, Japan).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Biopsies from scaffolds (one for every static/dynamic sample) with cultured cells were minced, immersed in 0.5 ml Trizol and stored (-80°C). RNA was isolated by

chloroform and isopropanol precipitation and treated with DNase I. RT-reaction was performed in Perkin Elmer thermal cycler using Super Script II Reverse Transcriptase according to the manufacturer's description (Life Technologies; Carlsbad, US). In brief, cDNAs were amplified by semi-quantitative real-time PCR using SYBR-Green PCR Master Mix (Roche; Basel, Switzerland) using a Light Cycler®480 Real Time PCR System. Finally, gene expression levels of alpha smooth muscle actin (ACTA2), calponin (CNN1), desmin (Desm) and type III collagen (Col3A1) were examined and HPRT (housekeeping gene) was used as a control (see Table 1 for primer sequences).

Immunohistochemistry

For histological evaluation, the scaffolds were embedded in Tissue-Tek (O.C.T. Compound) and snap frozen in dry-ice cooled isopentane. Cryostat sections (5 µm) were cut and fixed for 10 min in 100% acetone (-20°C) followed by a blocking step of 30 min with 10% (v/v) goat serum in 1% (w/v) bovine serum albumin/ phosphate buffered saline (BSA/PBS). Sections were incubated with one of the following mouse anti-human antibodies: alpha smooth muscle actin (α-SMA, Sigma-Aldrich; St. Louis, US; 1:8 000), desmin (BioGenex; Fremont, US; 1:200), calponin-1 (CNN1, Abcam; Cambridge, UK; 1:100), RCK103 (Cytokeratin 5 and others, Nordic MUbio; Susteren, The Netherlands; 1:1) and rabbit anti-bovine type I collagen (EMD Millipore, Germany; 1:100), all diluted in 1% (w/v) BSA/PBS, for 1 h. After washing (PBS, 3 times), sections were incubated with goat-anti-mouse-Alexa 594 (1:200) and goat-anti-rabbit-Alexa 488 (1:200) for 1 h. After rinsing with PBS (3 times), slides were incubated with 4',6-diamidino-2-phenylindole (DAPI; 1:200) for 20 min and 21°C. Finally, slides were mounted with fluorescent mounting medium (Dako; Glostrup, Denmark) and evaluated. Porcine bladder tissue was used as control tissue. Primary antibody was omitted as negative control. All anti-human primary antibodies had porcine cross-reactivity or were tested for cross-reactivity on porcine bladder tissue.

WST-1 proliferation assay

After culture, a transverse part of every scaffold (10x3 mm) was incubated in 500 µl medium with 50 µl cell proliferation reagent WST-1 (Roche; Basel Switzerland). After 2 h the absorbance at 450 nm was determined.

Results

Characterization scaffold

Scanning electron microscopy (SEM) analysis of the type I collagen scaffold showed a highly porous and interconnective network with pores ranging between 50 - 100 µm for both the air and pan side (Fig. 1c and d). The degree of crosslinking of the scaffolds was 72 ±12% by TNBS analyses.

Characterization of bladder-derived smooth muscle and urothelial cells

Primary urothelial cell cultures formed cobblestoned epithelioid monolayers. Phenotypic analysis revealed a homogeneous RCK103-positive cell population (Cytokeratin 5 and others) (Online Resource 1a and b). Isolated SMC showed a typical spindle cell shape and expressed α-SMA (Online Resource 1c and d), smoothelin, and desmin over a number of passages. UC contamination in the SMC culture was negligible as judged by RCK103 staining. There were no SMC present in the used UC culture.

Evaluation of bioreactor cultured scaffolds

Microscopic evaluation (H&E staining) of the constructs revealed a much denser SMC layer on the scaffold surface when uniaxial strain was applied (Fig. 2a and b) compared to static culture conditions. Phenotypic analysis showed α-SMA expression, regardless of culture conditions. However, relatively more α-SMA positive cells were present in the SMC seeded scaffold cultured under dynamic conditions compared to static culture conditions (Fig. 2c and d). The immunohistochemical analyses demonstrated more intense desmin and calponin1 staining in the dynamic cultured SMC (Fig. 2e, f, g and h). Although a limited number of data is available, the RT-PCR data showed similar results, with a trend of higher expression of ACTA2, CNN1 and desmin levels in dynamic cultures (Fig.3). WST-1 cell proliferation assays of the SMC seeded scaffolds cultured under dynamic conditions showed increased cell proliferation compared to static cultures (Fig. 4) SEM analysis revealed SMC alignment when the seeded construct was exposed to mechanical stimulation (Fig. 5a and b).

UC lined the honeycomb and lamellae structures of the scaffold more prominent when cultured under static conditions compared to the UC cultured under dynamic stress (Fig. 6). Phenotypic analysis revealed strong expression of RCK103 regardless of the culture conditions (Fig. 6c and d). As expected, urothelial cells did not express any of the determined markers (data not shown). WST-1 showed no differences in UC proliferation between static and dynamic culture. Finally, SEM analysis showed more ruptures in the confluent UC layer which was exposed to uniaxial strain, compared to the static cultures (Fig. 5c and d).

Discussion

One of the biggest challenges in tissue engineering is to create (preconditioned) templates that adequately mimic the native tissue at the phenotypic and organizational level. For bladder tissue engineering, cell seeded bladder dome-shaped scaffolds have been used in patients with variable results [13]. Until now, no method has proven to generate superior functional scaffolds for bladder tissues. Mechanical stimulation during scaffold preparation may improve the functional outcome. It has been used to condition engineered tissue based on the assumption that mimicking the physiological conditions of the native tissue improves its function once implanted to correct or replace lost tissue. In this study we show that mechanical stimulation of a porcine cell seeded collagen scaffold using cyclic uniaxial strain, mimicking the filling and emptying of the bladder

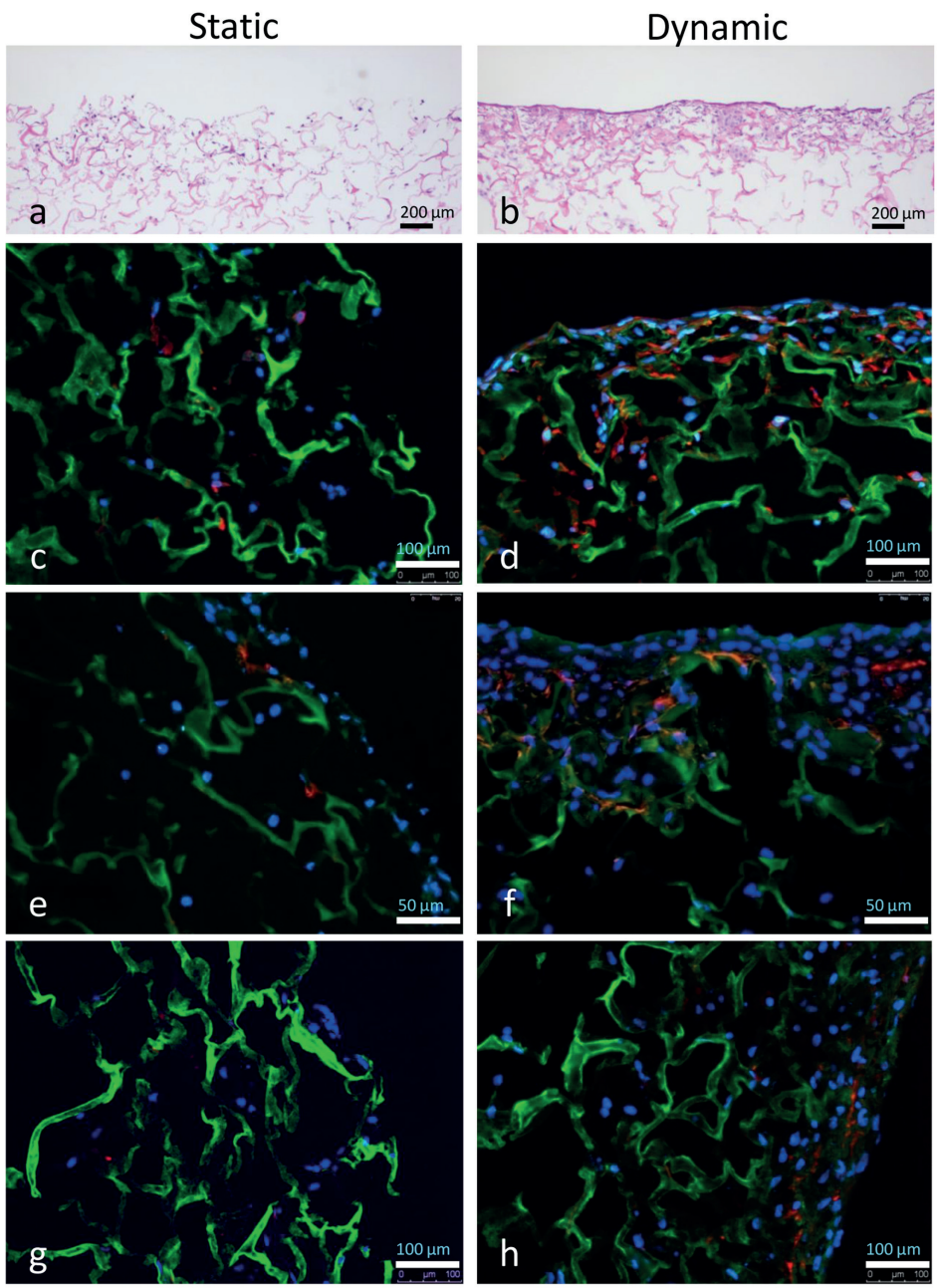

Figure 2. Representative Hematoxylin & Eosin (H&E) staining and immunofluorescence staining of the smooth muscle cell (SMC) seeded constructs cultured under static (a, c, e, and g) and dynamic (b, d, f, and h) conditions. a and b H&E staining, c and d α-SMA, E and F Calponin1 and g and h Desmin staining. Green: collagen, blue: nuclear DAPI stain, red: cell surface marker.

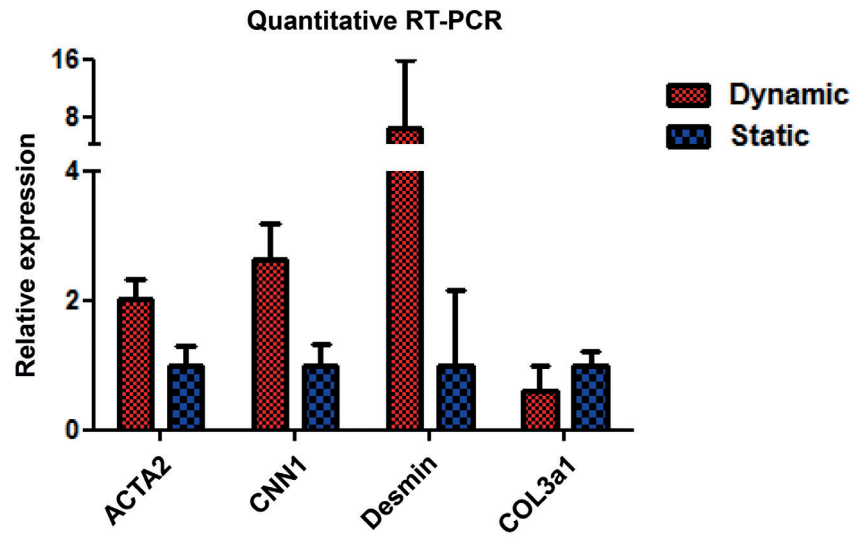

Figure 3. Quantitative RT-PCR data for α-SMA (ACTA2), calponin1 (CNN1), desmin (Desm) and collagen (Col3A1) of smooth muscle cell (SMC) seeded scaffold which were cultured under static and dynamic conditions. The relative expression of the different scaffolds was corrected for the internal HPRT control and the static conditions were set to 1. Bars represent the mean ± SD for 3 (SMC) separate experiments. None of the tested markers was expressed by the UC.

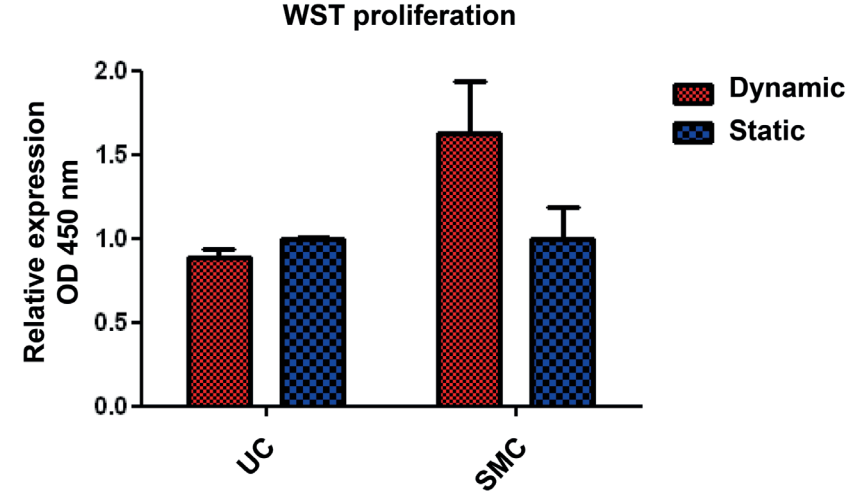

Figure 4. Proliferation (WST-1 cell proliferation assay) of urothelial cell (UC) and smooth muscle cell (SMC) seeded scaffold which were cultured under static and dynamic conditions. The relative expression of the static conditions were set to 1. Bars represent the mean ± SD for 2 (UC) or 3 (SMC) separate experiments.

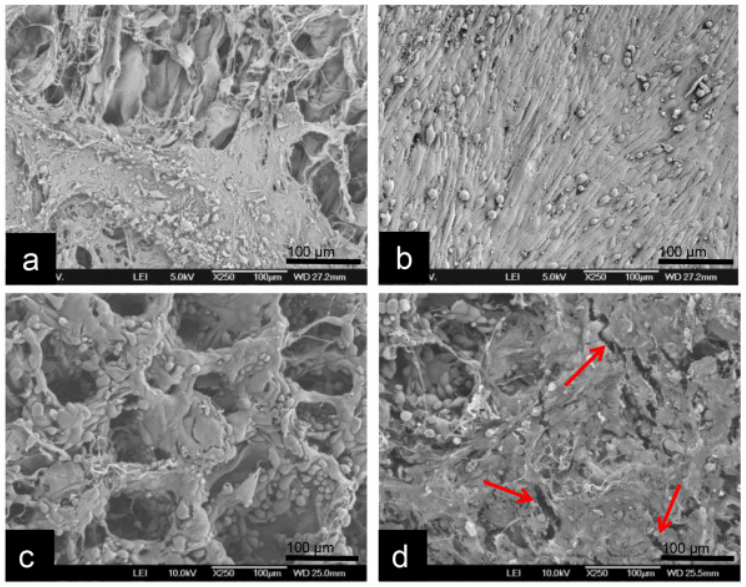

Figure 5. Representative scanning electron microscopy (SEM) analysis of the smooth muscle cell (SMC) and urothelial cell (UC) seeded constructs. a and b SEM of SMC cultured under static (a) and dynamic (b) conditions. c and d SEM of UC cultured under static (c) and mechanical condition (d). Please note aligned SMC in (b). Red arrows point to small ruptures (d)

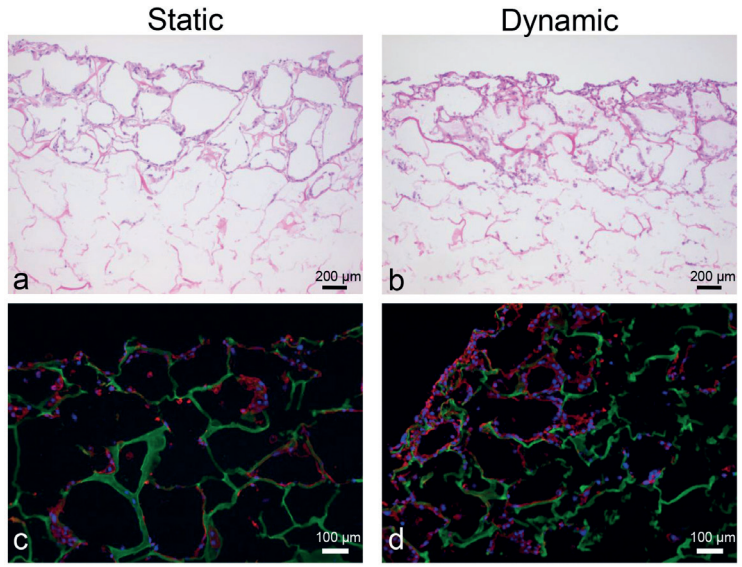

Figure 6. Representative Hematoxylin & Eosin (H&E) staining and immunofluorescence staining of the urothelial cell (UC) seeded constructs cultured under static (a and c) and dynamic (b and d) conditions. a and b H&E staining and c and d cytokeratin staining. Green: collagen, blue: nuclear stain (DAPI), red: cytokeratin.

results in increased smooth muscle cell growth, improved cell distribution, and more importantly SMC alignment, mimicking SMC organization in muscle fibers. Southgate et al. [14] showed that pig and human bladders have anatomical and biological similarities, making porcine derived bladder cells an adequate model for developing tissue engineering strategies.

Collagen-based scaffolds have been used extensively for bladder tissue engineering in (pre)clinical studies: immune responses are lacking and this natural biomaterial is highly cyto- and biocompatible. Nevertheless, despite these favorable characteristics implementation of collagen-based scaffolds is hampered by the poor and slow ingrowth of SMC after implantation [15, 16]. Whereas collagen scaffolds are replaced by newly formed tissue in 3-4 weeks, SMC ingrowth is limited to the rim of the defect. Enhanced interconnectivity of collagen scaffolds by decreasing the collagen density can lead to an improved SMC distribution [17]. Here we show that another possibility to improve SMC distribution is scaffold conditioning through pre-seeding and dynamic culturing of the construct. The current stimulation protocol was based on a pilot experiment where we compared slow, fast and “bladder like” stimulation protocols. The cellular distribution and cell density were superior. Most likely, the rapid stretch and or release induced too rapid material deformations, leading to cell detachment. Since we are using this technique to prepare in vitro conditioned scaffolds to function in the bladder, it may be that a “bladder like” protocol would be more effective. A possible refinement to prevent cell loss could involve a distinct filling (stretch) and extended emptying (release) phase.

Conditioning of the seeded construct by uniaxial stimulation resulted in SMC alignment. Moreover, SMC growing under mechanical stimulation showed higher α-SMA, desmin and calponin1 expression compared to static cultures as judged by PCR. Moreover, α-SMA, desmin and calponin1 levels were higher by immunohistochemistry. This is in agreement with Ahvaz et al. [18] who also observed increased α-SMA and calponin1 expression in SMC which were subjected to continuous stretch-relaxation cycles. Remarkably, the same investigators reported decreased α-SMA and calponin1 expression using a different (synthetic) substrate [10]. In experiments lasting 6 hours, calponin1 and α-SMA were down regulated during smooth muscle cell stimulation [19]. These discrepancies may be related to differences in cell source, passage number, medium type, mechanotransduction frequencies or physical and chemical properties of the scaffolds.

It has been hypothesized that mechanical stimuli also trigger surface stretch receptors and adhesion sites of cells, resulting in the activation of genes which are responsible for the synthesis and secretion of extracellular matrix components, such as collagens [20]. Since we used bovine derived collagen as a template for our cells, we were unable to investigate the production of type I collagen. When we analyzed type III collagen production, we did not observe any influence of culture conditions on type III collagen expression. This contrasts with the observation that in static cultures and non-compliant bladders the type III collagen production is decreased [21, 22]. It is possible that longer culture times are required to observe

increased collagen production. Other investigators have demonstrated that after longer culture periods (up to 14 days) type I and III collagen production levels increased in dynamic cultures [23].

The urothelium has an important osmotic barrier function: it protects the submucosa from environmental harm, mainly the toxic components of the urine. In our studies minor morphological and proliferation differences were observed between static and dynamic cultures, albeit that small cracks were present in the dynamic cultured urothelium. The small distortions that we observed in the UC layer are most likely due to the strain exerted on the collagen. Apparently SMC can accommodate this strain better than the UC. The absence of an adjacent lamina propria and/or fibroblasts in our in vitro model may indeed explain our observations. In the urinary bladder the UC are anchored on this superficial layer which contains both type IV collagen and elastin. Construction of collagen scaffolds in which UC and SMC are intimately connected will require a longer culture period. Alternatively, myofibroblasts could be used or dynamic culturing of SMC followed by a static culture of UC.

Augmentation cystoplasty with gastrointestinal tissue is a relatively safe and effective way to restore bladder capacity. However, this treatment can be associated with several complications including infections, stone formation, metabolic abnormalities and carcinogenesis. Tissue engineering may provide an alternative approach, but current methods have shown substantial side effects and suboptimal results. This may be related to the lack of mechanical stimulation during preparation, which can lead to fibrosis and poor compliance [13, 24]. The bladder is continuously filled and emptied, resulting in continuous loading of the tissue. By mimicking these dynamics during preparation of tissue engineered constructs a better outcome may be achieved. In addition, scaffold survival is heavily dependent on the formation of a new vascular bed. This is especially important when considering that large constructs are needed for relevant augmentation. To solve this, multiple smaller scaffolds instead of one large scaffold may be used (Roelofs et al., submitted). Thus, implantation of multiple conditioned scaffolds may be an attractive alternative for bladder augmentation using gastro-intestinal tissue; an assumption that needs to be tested in patients.

A potential drawback of the current study is that we only examined the effects of linear stretching on the cells while bladder filling causes a multi-directional strain. Whether multidirectional conditioning leads to a different outcome is unclear and bioreactor experiments along these lines may be of use. Moreover, a more physiological filling and contraction profile may be needed to create an optimally conditioned template. Finally, healthy bladder cells may not be available, and a different cell source may be required. Recently, stem cells derived from urine or adipose tissue have been successfully differentiated to functional smooth muscle cells for bladder tissue engineering applications. In addition, these stem cells can be harvested from the patient relatively easy, providing an autologous cell source [25, 26].

In summary, our results show that conditioning of collagen-based scaffolds by mechanical stimulation leads to more SMC with a more differentiated phenotype which may bypass difficulties related to poor SMC in-growth and muscle development in tissue engineered bladders. This brings us closer to our goal to engineer flexible tissues such as urinary bladder as an alternative to current graft tissues.

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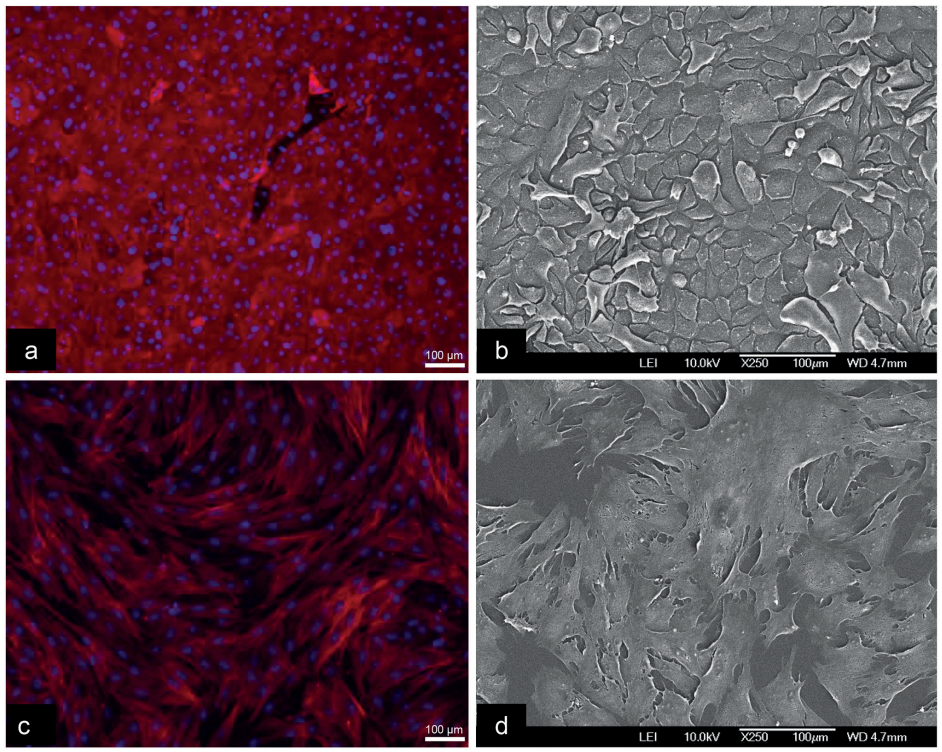
Appendix 1



Online Resource 1. Representative immunofluorescence staining and Scanning Electron Microscopy (SEM) of isolated porcine urothelial cells (UC) and bladder smooth muscle cells (SMC). UC stained with RCK103 (a), and αSMA expressing SMC (c) demonstrating homogenous cell populations. Scanning electron microscopic pictures of the used UC (b) and SMC (d) (250x)

Chapter 6

Bladder Regeneration using Multiple Acellular Scaffolds with Growth Factors in a Bladder

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Abstract

Introduction: Tissue engineering may become an alternative to current bladder augmentation techniques. Large scaffolds are needed for clinically significant augmentation, but can result in fibrosis and graft shrinkage.
The purpose of this study was to investigate the use of multiple scaffolds instead of one large scaffold, to enhance bladder tissue regeneration and bladder capacity. Secondly, acellular collagen, collagen-heparin, and collagen-heparin scaffolds with growth factors were used and the biological activity of the different scaffolds was compared in a large animal model.

Materials and methods: Scaffolds were made of bovine type I collagen with or without heparin (Ø = 3.2 cm). Collagen-heparin scaffolds were loaded with growth factors VEGF, FGF2 and HB-EGF. Three identical scaffolds prepared from collagen (COL-group), collagen with heparin (COLHEP-group), or collagen-heparin with growth factors (COLHEPGF-group) were implanted in one porcine bladder. The outcome was compared with sham operated animals (Sham-group), in which no scaffold was used. Urodynamic evaluation was performed prior to surgery and 3 months after bladder reconstruction, together with histological evaluation.

Results: Survival rate was 92%, 12 animals completed the study, 3 of every group, 1 animal developed peritonitis due to urine leakage and was sacrificed. The regenerated area was largest in the COLHEP-group, and least in the COL-group (p=0.002). Histological evaluation revealed a normal urothelial layer and good angiogenesis in all groups, and comparable ingrowth of smooth muscle cells.
Urodynamics showed no statistically significant differences in bladder capacity and compliance between groups. Bladder capacity and compliance was very high in this animal model, which made it impossible to study the increase due to augmentation.

Conclusions: Implantation of multiple collagen-heparin scaffolds in one bladder is feasible in a porcine model, resulting in tissue almost indistinguishable from native tissue involving all cell layers of the bladder. Collagen scaffolds with heparin incorporated resulted in a larger area of regenerated tissue. To reach clinically significant augmentation, multiple larger collagen-heparin scaffolds, with or without growth factors, need to be tested to study the largest possible diameter of scaffold and number of used scaffolds still resulting in well vascularized tissue.

Introduction

Bladder augmentation is often indicated in children whose bladder has a small capacity or low compliance. The main causes are congenital anomalies such as bladder exstrophy, a neuropathic bladder caused by a myelomeningocele, or posterior urethral valves.1 Gastrointestinal tissues are most often used to augment the bladder. However, the use of gastrointestinal tissues can be problematic: it is not always sufficiently available, and its use can lead to metabolic disturbances, infections, excessive mucus production, stone formation, perforation and even malignancies [1-4]. Several studies have shown that tissue-engineered constructs can be used to regenerate bladder tissue, including urothelium, smooth muscle, vessels and nerve fibers, in various animal models [5-7]. A substantial improvement in bladder capacity was found in the first clinical trial by Atala et al., who showed the feasibility of tissue engineering of the human bladder. Particularly promising results were seen with constructs consisting of collagen and a synthetic polymer, seeded with urothelial and smooth muscle cells (SMCs), and wrapped in omentum [8]. Unfortunately, these results could not be reproduced in a phase 2 study in which 10 patients with spina bifida were treated with this technique [9]. Augmentation of the bladder of five patients with bladder exstrophy with an acellular small intestinal submucosa (SIS) scaffold improved bladder capacity. However, the primary clinical endpoint of improved dry intervals was not reached [10]. These studies clearly show that improvements of the technique are needed to enhance clinical outcome, which is one of the essential steps which need to be undertaken before it can be tested again in a clinical trial [11].

Tissue regeneration in large constructs is hampered by a lack of oxygen and nutrition delivery to the infiltrating cells and inadequate removal of waste products [12, 13]. The diffusion distance from supplying blood vessels is approximately 150-200 µm [13], necessitating rapid and extensive angiogenesis. In the current study the feasibility of implantation of 3 smaller scaffolds instead of one large scaffold, to reach the same surface area of regenerated tissue while reducing the problem of oxygen and nutrition delivery in large scaffolds, was examined. We hypothesized that the shorter distance of the vascularized border of the native tissue to the center of the scaffold would reduce the vascularization time of this area, and consequently the period of limited oxygen and nutrient delivery, and improve tissue regeneration.

Growth factors (GFs) play an important role in proliferation, migration and differentiation of several cell types. The inclusion of GFs in large grafts may therefore assist in quicker and better acceptance [12, 14, 15]. Vascular endothelial growth factor (VEGF), an important factor in angiogenesis, combined with fibroblast growth factor 2 (FGF2) enhances blood vessel formation and maturation [12]. Collagen scaffolds loaded with heparin were used to bind these GFs, together with heparin-binding epidermal growth factor (HB-EGF) which is known to play a role in urothelial regeneration [14-17]. This combination of growth factors improved regeneration of bladder tissue in a large animal model for diseased bladder, resulting in better ingrowth of urothelium and SMCs, and enhanced angiogenesis [15]. In the current study collagen-heparin scaffolds loaded with

these growth factors were compared to a collagen scaffold, a collagen scaffold with heparin alone, and a sham-operated control group.

Materials and methods

Preparation and characterization of scaffolds

Type I collagen was purified from bovine Achilles tendon as previously described [18]. A 0.67% (w/v) type I collagen suspension in 0.25 M acetic acid was shaken overnight at 4ºC and homogenized on ice using a Potter-Elvehjem homogenizer (Louwers Glass and Ceramic Technologies, Hapert, The Netherlands). Air bubbles were removed by centrifugation at 100g for 15 min at 4ºC. The suspension was poured into 6-well plates (4 mL per well), frozen at -20ºC, and lyophilized. For cross-linking, scaffolds were pre-incubated with 50 mM 2-morpholinoethane sulfonic acid (MES) pH 5.0 containing 40% ethanol for 30 min at 20ºC. After removal of this solution, scaffolds were incubated with 33 mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and 6 mM N-hydroxysuccinimide (NHS) in 50 mM MES pH 5.0 containing 40% ethanol for 4 h at 20ºC with or without 0.25% heparin (Diosynth, Oss, The Netherlands). Scaffolds were washed, frozen and lyophilized. This resulted in round collagen scaffolds with a diameter of 3.2 cm. Subsequently, 70% ethanol was used to disinfect the scaffolds, followed by washings with sterile PBS. To bind the GFs, collagen-heparin scaffolds were aseptically incubated with FGF2, VEGF and HB-EGF (all human recombinant; R&D Systems, Minneapolis, MN, USA). First, scaffolds were incubated in 7 ml PBS containing 3.5 µg/ml FGF2 for 1 h. Next, VEGF and HB-EGF, each 3.5 µg/ml PBS, were added and incubated overnight at 20°C. Finally, scaffolds were washed in PBS and used immediately.

The number of primary amine groups was analyzed using a 2,4,6-trinitrobenzene sulfonic acid assay to determine the degree of crosslinking [19, 20]. Heparin content was determined by a hexosamine assay with p-dimethylamino-benzaldehyde, using a standard curve of heparin [21]. GF content was assessed using Western blot analysis [14]. Scaffold ultrastructure was visualized using a JEOL JSM-6310 scanning electron microscopy (SEM) apparatus by operating at 15 kV, after gold-coating the specimens.

Surgical procedure

This study was approved by the Animal Ethics Committee of the Radboud university medical center.

Thirteen female Landrace pigs with a mean weight of 48.5 kg (ranging from 41-59 kg) were operated at age 3 months. No significant difference in weight existed within and between groups. Animals were housed individually for two weeks after surgery, with a restricted diet and free access to water. After two weeks the animals were housed in a group. Animals were pre-medicated with intramuscular (IM) injection of midazolam (1 mg/kg, Roche, Woerden, The Netherlands), atropine (50 µg/kg, Pharmachemie BV, Haarlem, The Netherlands) and ketamine (10 mg/kg, Eurovet Animal Health BV, Bladel, The Netherlands). General anesthesia was induced by intravenous (IV) injection of propofol (2-3 mg/kg, B. Braun, Melsungen, Germany), followed by tracheal intubation, and maintained with 0.5-1% isoflurane (Nicholas Piramal, London, UK) and midazolam (0.6 mg/kg/h, IV). For analgesia, flunixin (2.2 mg/kg, Intervet, Boxmeer, The Netherlands) and sufentanil (5 µg/kg bolus, Janssen Cilag BV, Tilburg, The Netherlands) were given IV, followed by a maintenance dosage sufentanil of 10 µg/kg/h. Vecuronium (0.2 mg/kg, N.V. Organon, Oss, The Netherlands) was given IV as muscle relaxant, and maintained with 0.4 mg/kg/h.

Video urodynamic evaluation was performed using the MMS Solar system (MMS, Enschede, The Netherlands) and a Philips BV-25 C-arm and image identifier (Philips, Eindhoven, The Netherlands). A double lumen 6Fr catheter (Medtronic, Heerlen, The Netherlands) was placed in the bladder. A double lumen 9Fr catheter (Bel Medical B.V., Zwolle, The Netherlands) was placed in the rectum. The bladders were slowly filled with iodinated contrast fluid (Xenetix® 300 (Guerbet Nederland BV, Gorinchem, The Netherlands) diluted with PBS 1:1). Urethral leakage did not occur during urodynamic measurements, so bladder filling was stopped at a maximal pressure of 40 cm H2O and bladder capacity was evaluated at 20 cm H2O. One or two representative evaluations were performed in each animal.

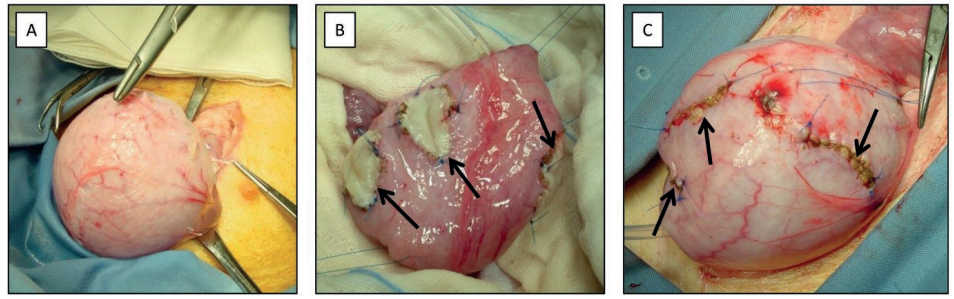

Figure 1. Representative macroscopic overview of the implantation procedure. A. The bladder was exposed and 3 cm long incisions were made. B. In the experimental groups collagen scaffolds were sutured into the defects. C. In the control animals the defects were primarily closed.

Subsequently a laparotomy was performed under sterile conditions to expose the bladder (Fig. 1a). Three incisions of 3 cm were made in the bladder, along the vascularization from the lateral side to the middle of the bladder. Animals were randomly assigned to one of the four groups. Three identical collagen scaffolds (COL-group), collagen-heparin scaffolds (COLHEP-group), or collagen-heparin scaffolds with growth factors (COLHEPGF-group) were sutured into the defects using 5-0 poliglecaprone (Monocryl®, Ethicon Inc. NJ, USA) running sutures (Fig. 1b). In the sham-group the bladder was closed in one layer using a 5-0 poliglecaprone running suture (Fig. 1c). Four 5-0 polypropylene (Prolene®, Ethicon Inc.) nonresorbable marking sutures were placed at the edges of the scaffold. A 12Fr silicon catheter (Coloplast, Amersfoort, The Netherlands) was used as suprapubic catheter. The bladder was filled to ensure that no urinary leakage occurred along the suture line. The peritoneum was closed using 4-0 poliglecaprone. The

abdominal wall was closed using 0 polyglactin (Vicryl®, Ethicon Inc.) interrupted sutures for the fascia and 1-0 polyglactin for the skin. The suprapubic catheter was fixed to the skin using 1-0 polyglactin sutures, and was removed after 2 weeks. Buprenorphine (10 µg/kg, IV, Schering Plough, Segre, France) was given once, and flunixin (2.2 mg/kg, IM) for three days as postoperative analgesia. Amoxicillin (10mg/kg, IM, Aurobindo Pharma BV, Zwijndrecht, The Netherlands) was given twice daily for 3 days.

Functional evaluation

Animals were evaluated after 3 months. Animals were anesthetized and urodynamic evaluation was performed as described above. Thereafter the animals were sacrificed with IV pentobarbital (60 mg/kg, AST Pharma, Oudewater, The Netherlands). The bladder was removed, filled with 300 ml PBS, inspected, and the size of the regenerated tissue was measured between marking sutures (Fig. 2). The regenerated area was assumed to be of elliptical shape, the surface area was calculated using the formula (0.5 x diameter 1) x (0.5 x diameter 2) x π. Kidneys were examined for signs of infection, hydronephrosis, or stone formation.

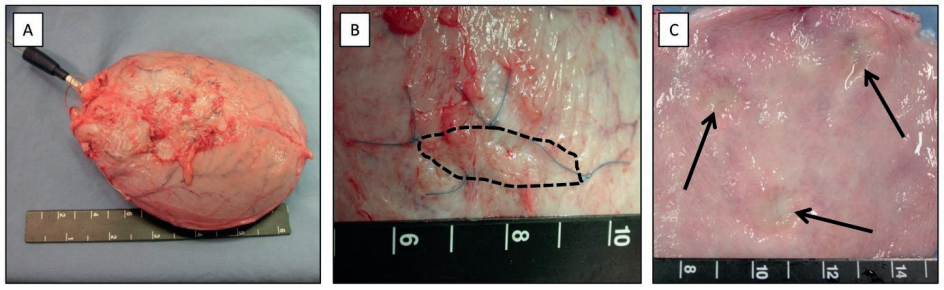

Figure 2. Macroscopic overview 3 months post-surgery. A. The bladders were filled with 300 ml of saline to ensure equal exposure between samples for measurements. B. Implantation sites were recognized by the four marking sutures. C. The luminal side of the bladder was exposed, showing the three areas where the scaffolds were implanted (arrows).

Histological staining

Tissue samples were obtained from the regenerated tissue (COL-group, COLHEP-group and COLHEPGF-group) and the scar tissue (sham-group), fixed in 4% (v/v) buffered formalin and paraffin-embedded. Sections (4 µm) were cut in the center of the tissue along the longest axis and stained with hematoxylin & eosin (H&E). Multiple levels were analyzed using H&E staining and a representative level was chosen for immunohistochemistry and the Masson trichrome/Verhoeff stain. For immunohistochemistry, sections were deparaffinized in xylene, followed by graded series of ethanol and re-hydration in PBS. Slides were immersed in 1% (v/v) H_2O_2/PBS for 30 min at room temperature to block endogenous peroxidase. Antigen retrieval methods and primary antibodies are shown in Table 1. Slides were pre-incubated with 5% goat serum, incubated with the antibody of interest: pancytokeratin , Cytokeratin 7, smooth muscle actin or smoothelin, washed and incubated with poly-HRP-anti mouse IgG (Immunologic, Duiven, The Netherlands) which was pre-incubated with 10% swine serum for 30min at RT. For vimentin and desmin staining, slides were peroxidase blocked and pre-incubated with 5% horse serum. After primary antibody incubation for 1.5 hour, sections were washed and incubated with a biotin-labeled secondary antibody (30 min, horse-α-mouse, 1:200, Vector laboratories, Burlingame, California, USA). Next, the slides were washed and incubated with peroxidase-labeled streptavidin/biotin complex (1:100 Avidin reagent and 1:100 Biotin reagent, 30 min pre-incubation, Vector Laboratories, Burlingame, California, USA) for 45 min followed by washings in PBS. All stainings were developed with Bright-DAB (Immunologic) for 10 min and counterstained with hematoxylin (Klinipath, Duiven, The Netherlands).

Histological evaluation was performed microscopically by LR, PdJ and PG and scored independently in a blinded fashion. Quantitative analysis of smooth muscle cell ingrowth was performed using ImageJ (NIH, USA). Using a 'rolling ball method' (50 px), background was corrected for desmin stained slides. The browns were extracted and converted to binary, followed by plotting the pixel intensity along the native muscle and the regenerated tissue. The intensity thereby is higher when more muscle tissue is present (Fig. 3). The distance of muscle ingrowth was determined. In addition, the area under the curve (number of positive pixels, derived from curves as shown in Fig. 3) was analyzed to approximate the amount of muscle tissue in the regenerated and native tissue. This was standardized to show the average amount of pixels per mm of tissue (AUC / mm).

Renal tissue of all pigs was processed and examined for inflammation or dilated nephrons by H&E staining.

Statistical analysis

Data analysis was performed with IBM SPSS statistics version 22 (IBM, New York, USA), using the one-way ANOVA test, followed by the LSD post hoc test. $P < 0.05$ was considered statistically significant.

Results

Scaffolds

The collagen scaffolds consisted of homogenously distributed honeycomb-like pores with an average size of 100-150 µm (see Hosper et al [22]. who used identical scaffolds). After crosslinking, primary amine groups were reduced by 46 ± 6% for collagen scaffolds and 48 ± 8% for heparinized collagen scaffolds. Heparin bound to heparinized collagen scaffolds was 12 ± 5%. The GF content bound per mg collagen-heparin scaffold were 0.2 ± 0.2 µg for VEGF, 0.7 ± 0.2 µg for FGF2, and 0.8 ± 0.3 µg for HB-EGF. Results are given as mean ± SD.

Animal surgery

Overall survival rate was 92%. One out of 13 pigs had a peritonitis due to urinary leakage and was sacrificed. At autopsy we found a rupture of the scaffold at the border where it was sutured. One other animal was sacrificed one day before the intended date of sacrifice because of illness, no clear diagnosis was made after

autopsy. This animal was included in histological analysis. Both animals were from the COLHEPGF-group. Three months after operation the mean weight was 108.3 kg (range 90-120 kg), no significant differences existed within and between groups. This was an increase of 123% (Table 2).

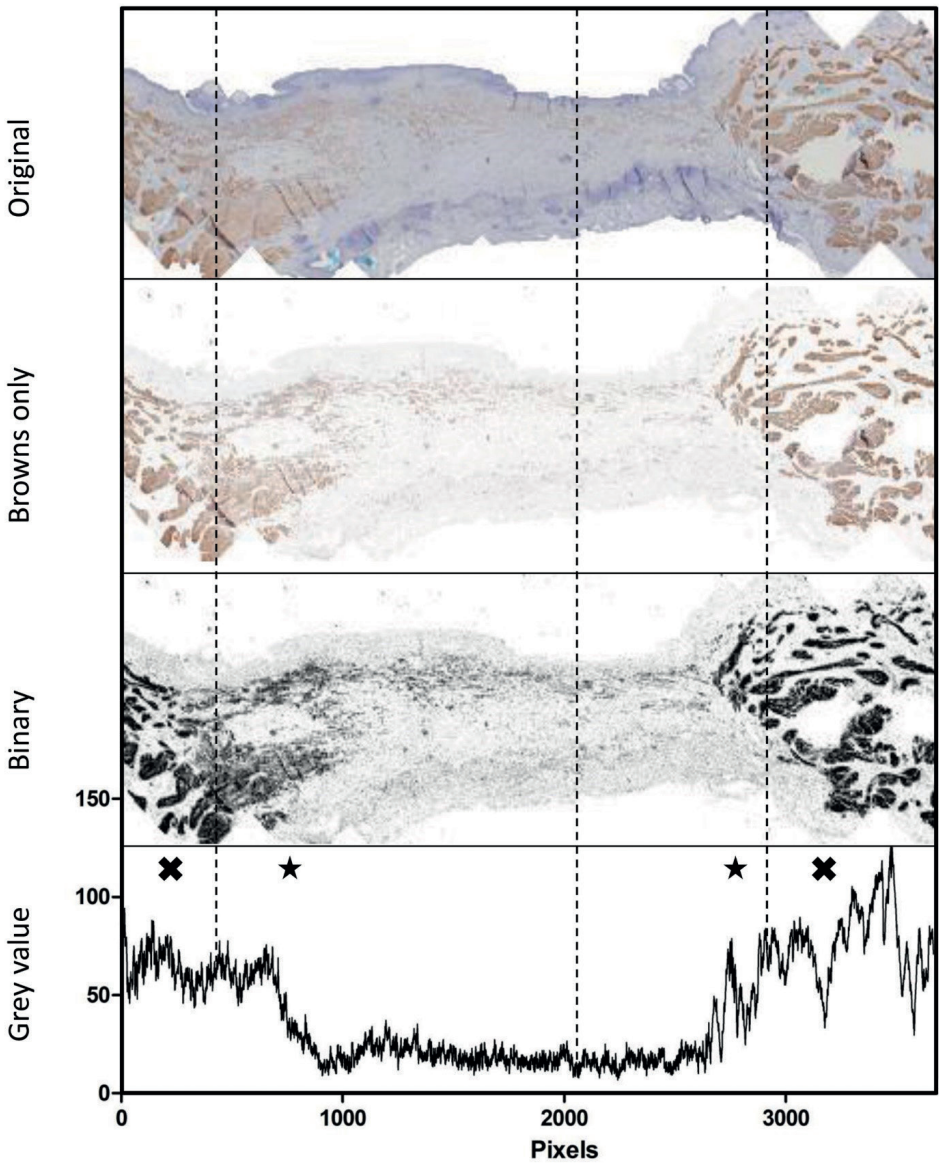

Figure 3. Overview of muscle quantification process. From the original image, only the brown colors (desmin positive cells) were extracted. Next, the image was converted to a binary image. Finally, a profile was plotted which showed the pixel intensity along the tissue. Higher intensity indicates a higher number of desmin positive cells. X indicates the native muscle tissue. * Indicates the anastomosis site. Note the change of morphology and decreasing intensity towards the center.

Functional evaluation

All pigs voided normally, without signs of incontinence or urinary tract infection. Catheterization was impossible in one animal before operation and two animals at 3 months. On urodynamic evaluation none of the pigs showed detrusor overactivity. No statistically significant differences in capacity were found between groups (Table 2).

No structural abnormality of the bladders, e.g. no diverticula or leakage were seen when the bladders were filled after sacrifice (Fig. 2a).

Macroscopic evaluation

No scaffold remnants were visible in the scaffold groups (Fig. 2b). The bladder wall appeared slightly thinner at the implantation site in these groups. A small scar was visible in the sham group. The native bladder had a normal appearance in all groups, without signs of stone formation (Fig. 2c). The sum of the regenerated area is shown in Fig. 4a and Table 2. The largest amount of tissue was regenerated in the COLHEP-group, which was statistically significant compared to the COL-group ($p=0.002$), but not compared to the COLHEPGF-group ($p=0.083$). No significant difference was seen between the COL-group and COLHEPGF-group ($p=0.215$). All kidneys appeared normal.

Histological evaluation

Figures 5-7 show representative sections from the different study groups.

Sham group

A confluent, normal appearing multilayered lining of urothelial cells was observed, containing a basal layer and umbrella cells. The architecture of the tissue was generally like native tissue, including smooth muscle formation. Only few fibroblasts and myofibroblasts were found in the wound (data not shown).

Regenerated bladder tissue in scaffold groups

The histological results are shown in Table 3. The regenerated tissue showed a confluent, well differentiated, urothelial layer in all groups (Fig. 5a,b, 6a,b and 7a,b) (data cytokeratin 7 not shown). Submucosal tissue consisted of connective tissue with fibroblasts, myofibroblasts and dense collagen (Fig. 5a,d,e; 6a,d,e; 7a,d,e). Directly under the urothelium the multilayered structure of vimentin- and α-SMA-positive cells, similar to native bladder tissue, was visible in approximately half, and all animals, respectively. No difference was seen between groups (Fig. 5d,e; 6d,e; 7d,e). Fibrosis was comparable between groups, and slightly more evident in the COLHEP-group, with slightly denser extracellular matrix (ECM) and slightly more fibroblasts and myofibroblasts. Angiogenesis was visible throughout the entire regenerated area and comparable between groups (Table 3). Mature blood vessels were present, evidenced by vimentin positive cellular linings and the presence of erythrocytes (Fig. 7G). In the COL-group and the COLHEPGF-group hardly any scaffold remnants remained. In the COLHEP-group scaffold remnants were visible, mainly at the serosal side (Fig. 5a,c, 6a,c and

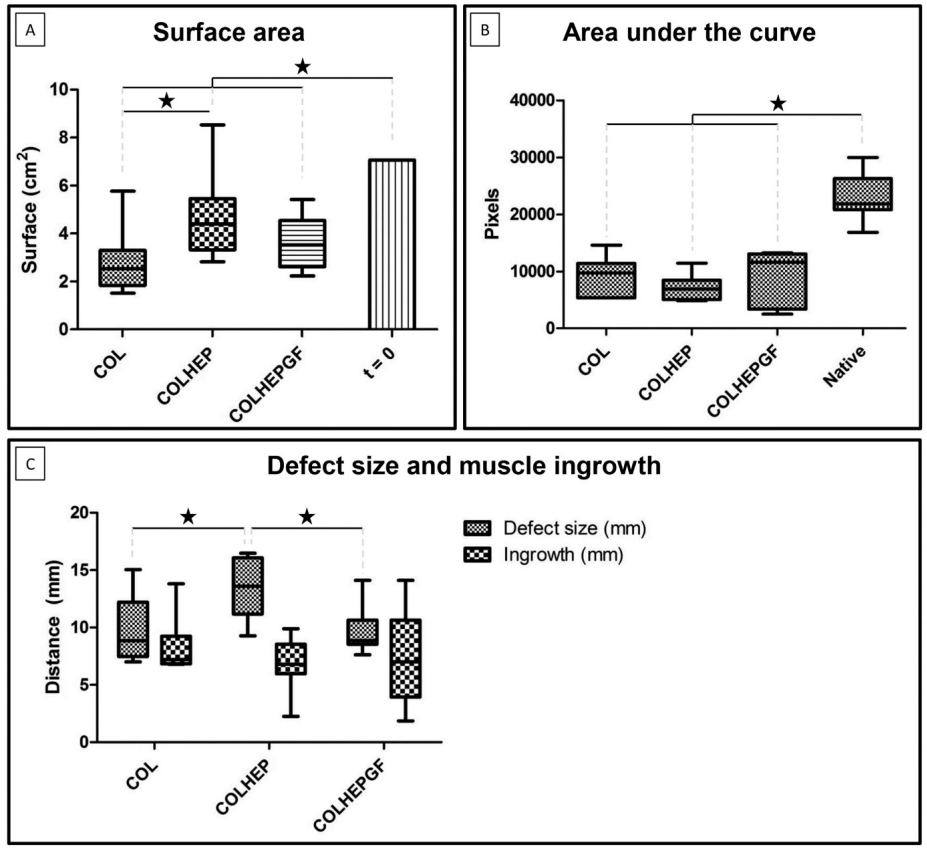

Figure 4. Results of the quantitative and statistical analysis. A. Macroscopic surface area (cm2) was determined. The surface area of COL (n=9), COLHEP (n=9) and COLHEPGF (n=6) were significantly smaller ($p < 0.001$ for all) than the implanted scaffolds. The surface area of COLHEP was significantly larger ($p = 0.002$) than COL but not larger than COLHEPGF ($p = 0.083$). No significant difference was seen between COL and COLHEPGF ($p = 0.215$). B. The AUC / mm (pixels) was determined as a measure for the amount of muscle present per mm tissue based on the number of positive pixels. In all cases significantly less muscle was found ($p < 0.001$) than in native muscle tissue, but no differences between groups were observed ($p > 0.05$). COL (n=7), COLHEP (n=8), COLHEPGF (n=6), native (n=12). C. Histological defect size was determined by quantitative analysis. COLHEP (n=8) was significantly larger than COL ($p = 0.01$, n=8) and COLHEPGF ($p = 0.003$, n=6). No significant differences were seen between COL and COLHEPGF ($p = 0.596$). No significant differences were found regarding the distance of muscle ingrowth ($p > 0.05$). Whiskers represent the min-max range in all figures.

7a,c). Inflammatory cells were mainly visible around scaffold remnants, mainly consisting of lymphocytes. Slightly more inflammatory cells were observed in the COL-group. SMC ingrowth was present throughout almost the entire regenerated tissue in most animals of the COL and COLHEPGF group, appearing as fascicles or separate cells. While the distance of ingrowth was similar between all groups, the ingrowth in the center of the new tissue was limited in the COLHEP group due to the larger overall surface area. (Fig. 5a,f, 6a,f and 7a,f) (data smoothelin not shown). SMC ingrowth occurred from the borders inwards, originating from the

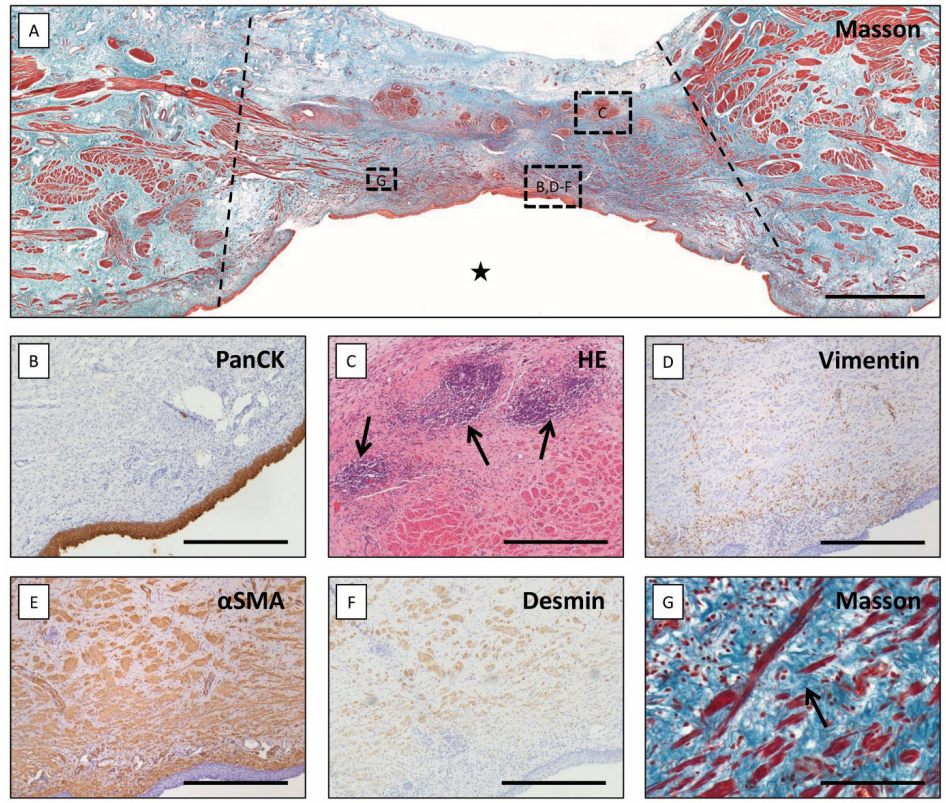

Figure 5. Representative histological overview of COL group after 3 months. A. Overview of the native and full regenerated tissue (between dashed lines). B. The luminal side was covered with a normal appearing multilayered urothelium. C. A moderate immune response (lymphocytes) could be observed locally near scaffold remnants. D. Only some vimentin positive cells were observed, indicating a well progressed regeneration process. E. Muscle tissue was observed in the middle of the regenerated tissue, with only few myofibroblasts. F. Desmin positive cells indicating maturing muscle tissue. G. Small elastic fibers were present in the newly formed tissue. Scale bars A = 2 mm, B-F = 500 um, G = 125 um. * indicates the lumen of the bladder.

native detrusor muscle. No clear difference in the amount of ingrowth of SMCs was visible between groups on microscopic evaluation. Quantitative analysis of smooth muscle ingrowth showed comparable number of SMCs per mm regenerated tissue (Fig. 4b). In view of the larger regenerated area of the COLHEP-group, a larger total amount of SMCs was present in the regenerated area of this group. The distance of ingrowth of these cells (when evaluating length of ingrowth from the anastomosis inwards) was comparable between groups (Fig. 4c).

Elastic fibers were scarcely found between the newly formed muscle fibers, and slightly more in the COLHEP-group (Fig. 5g) indicated by purple/black fibers in the Masson trichrome/Verhoeff stain. Nerve fibers were sporadically seen, only at the borders of the regenerated tissue, no differences between groups were seen (Fig. 6g).

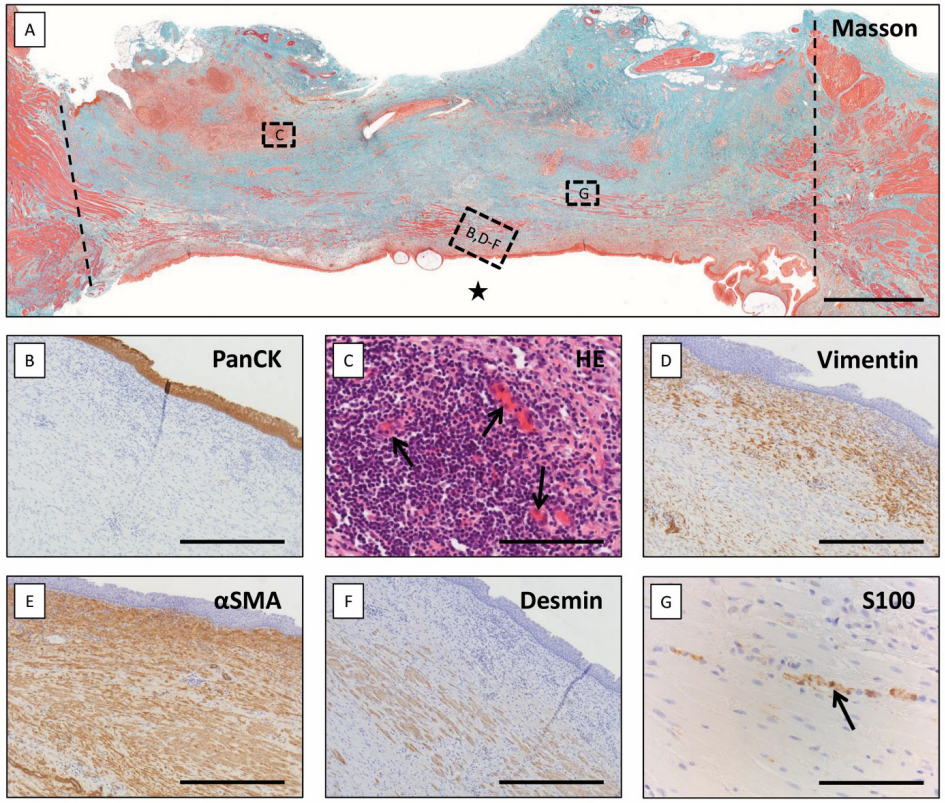

Figure 6. Representative histological overview of COLHEP-group after 3 months. A. Overview of the native and full regenerated tissue (between dashed lines). B. The luminal side was covered with a normal appearing multilayered urothelium. C. A moderate immune response (lymphocytes) could be observed near scaffold remnants (arrows). D. More vimentin positive cells were found in the COLHEP-group compared to the COL- and COLHEPGF-group. E. αSMA staining was also more intense in the COLHEP-group compared to the COL- and COLHEPGF-group. F. Desmin positive cells indicating maturing muscle tissue. G. Small nerve fibers could be observed in the regenerated tissue. Scale bars A = 2 mm, B,D-F = 500 um, C,G = 125 um. * indicates the lumen of the bladder.

Kidney tissue

No abnormalities were found in kidney tissue (data not shown).

Discussion

In this study we evaluated regeneration of bladder tissue in a large animal model and used multiple smaller scaffolds instead of one large scaffold. We showed the feasibility of this technique, and were able to regenerate bladder tissue that was almost indistinguishable from native bladder tissue with ingrowth of all essential components of the bladder wall and no necrosis in the scaffold areas. The urothelial layer was completely regenerated in all groups, vasculature was present in the whole tissue, and SMCs were growing into the regenerated area, as well as sporadic small nerve fibers.

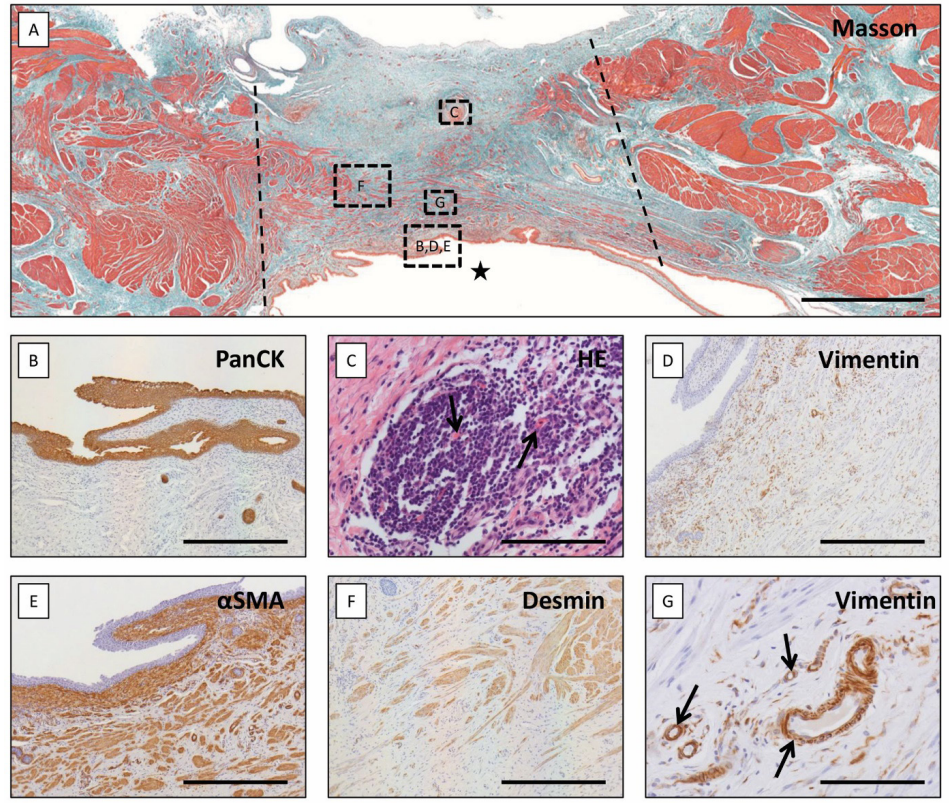

Figure 7. Representative histological overview of COLHEPGF group after 3 months. A. Overview of the native and full regenerated tissue (between dashed lines). B. The luminal side was covered with a normal appearing multilayered urothelium. C. A moderate immune response (lymphocytes) could be observed near scaffold remnants (arrows). D. Only some vimentin positive cells, indicating a well progressed regeneration process. E. Muscle tissue was observed in the middle of the regenerated tissue, with some myofibroblasts. F. Desmin positive cells indicating maturing muscle tissue. G. Extensive angiogenesis of small and larger vessels was observed. Scale bars A = 2 mm, B,D-F = 500 um; C,G = 125 um. * indicates the lumen of the bladder.

Macroscopic evaluation showed that the largest quantity of tissue was regenerated in the COLHEP-group. This was statistically significantly higher compared to the COL-group, but not compared to the COLHEPGF-group. The larger amount of regenerated tissue in the COLHEP-group might be explained by lesser contraction during regeneration due to the highly negative charge of the incorporated heparin with its sulfate and carboxylic groups. The negative charge may protect collagen from proteolysis, and has an inhibitory effect on the macrophage adhesion, hereby delaying degradation of the collagen scaffold which may reduce contraction [14, 23]. This also explains the larger amount of scaffold remnants at time of evaluation in the COLHEP-group. This slower degradation process of the collagen scaffold may have resulted in improved regeneration of bladder tissue due to a slower remodeling process. The larger number of myofibroblasts may explain the larger amount of collagen produced

in the regenerated tissue. Earlier evaluation is needed to determine whether myofibroblast content or delayed scaffold degradation cause the improved regeneration.

In contrast to previous findings, GF-loading did not show a beneficiary effect in this study when bladder regeneration was evaluated after 3 months. In previous work we observed improved ingrowth of urothelium, SMCs and improved vasculature 1 month after implantation with a collagen scaffold loaded with the same GFs as used in the current study [15]. In the previous study only a beneficial effect on SMC ingrowth was seen after 3 months [15]. This suggests that initially the GFs have a stimulatory effect on ingrowing cells, but this effect seems to wane. The availability and activity of growth factors most likely declined after a given time although GFs were bound to the scaffolds through the use of heparin, which also stabilizes GFs and protects them against proteolytic degradation, creating a sustained release system [24]. Pieper et al. studied the in vitro release profile of FGF2 from a collagen scaffold loaded with heparan sulfate in PBS [24]. Following an initial burst release of 13% at day 1, 53% was gradually released during 4 weeks of incubation. Kanematsu et al. showed similar release profiles of FGF2 loaded to a bladder acellular matrix (BAM) scaffold in PBS or urine in an in vitro study [25]. Furthermore, biological activity was maintained even after 4 weeks when implanted in the subcutis of mice [25]. Clearly, the subcutis and bladder are incomparable, in the bladder the implanted scaffolds are exposed to urine flow, which could increase the wash out of GFs. Data on in vivo release kinetics and activity of GFs incorporated in a collagen scaffold implanted in the bladder are lacking, and particularly important long-term results. Our results of the current and previous study suggest that the beneficial effects of GF loading can be achieved even in an environment with high urine flow, but that this effect is limited. Furthermore, COLHEP scaffolds may have attracted and bound growth factors from the wound environment and hereby improved regeneration. Indeed, heparin can bind a width variety of proteins and growth factors involved in wound healing, like TGF- β [ESKO][26]. However, by incorporating growth factors in a scaffold it is possible to guide regeneration in a favorable way. For instance, TGF-β plays an important role in wound healing, the isoform β1 was considered to increase fibrosis, while TGF-β isoform 3 may result in scarless wound healing. Incorporation of these growth factors may be optional, although the exact mechanism of TGF-β still needs to be elucidated [27].

In the current study we used 3 smaller scaffolds to reach the same regenerated surface area as with 1 larger scaffold. This decreases the area of tissue remodeling per scaffold to overcome the problem of angiogenesis in large scaffolds. Hereby, the vascularized border of the native tissue will be closer to the center of the scaffold, shortening the time until this area will be vascularized and reducing the distance for cellular ingrowth. It may be possible to implant more than 3 scaffolds to improve the augmentation of the bladder. However, one should take in account the sideways alignment of bladder vasculature, which may mean that scaffolds cannot be implanted next to each other, otherwise regeneration may be hampered by insufficient delivery of nutrients and oxygen for regeneration. We used scaffolds of 3.2 cm in diameter to make this study comparable to previous work in which we also used scaffolds of this size [15, 28, 29]. The normal functional capacity of a human bladder is approximately 300 ml, at which the bladder has a surface area of 216 cm2. The total area of implanted scaffolds was 21 cm2, which is 10% of the functional capacity of a normal human bladder. To reach clinically significant augmentation, the largest possible diameter and number of scaffolds still resulting in well vascularized tissue need to be studied.

With larger scaffolds, the strength of the scaffolds may need to be improved. In the current study we had one animal with urinary leakage due to a rupture of the scaffold at the side of the sutures. Inclusion of a degradable polymer may be a possible solution. Different polymers have already been tested for this purpose, with favorable results of a combination of collagen with Vicryl [30]. Additionally, incorporation of autologous urothelial and smooth muscle cells may improve tissue regeneration. However, harvesting, culturing and seeding of cells is time and cost consuming. Furthermore, when these cells are harvested from diseased bladders they may behave dissimilar to normal cells, making their use for tissue engineering questionable [29].

We used a porcine model because pigs are comparable sized to humans, have similar abdominal anatomy and results of smaller animal models often cannot be extrapolated to humans [31]. Urodynamic evaluation showed very high bladder capacities in the studied animals, and it was not possible to study the increase of capacity due to the augmentation. Tu et al [32]. found comparable urodynamic outcomes in their porcine study on bladder tissue engineering. We conclude that the porcine model is inappropriate for studying bladder augmentation by tissue engineering due to the very high compliance and capacity of porcine bladders. Alternatively, goat or sheep models may be more useful for this purpose [31].

Conclusions

Implantation of multiple collagen-heparin scaffolds for bladder augmentation may be a good alternative compared to one large scaffold. We show that this approach is feasible in a porcine model, resulting in tissue almost indistinguishable from native tissue involving all cell layers. Collagen scaffolds with heparin incorporated resulted in a larger area of regenerated tissue, which was statistically significant when compared to the COL-group. To reach clinically significant augmentation, multiple larger collagen-heparin scaffolds, with and without growth factors, need to be tested to study the largest possible diameter of scaffold and number of used scaffolds still resulting in well vascularized tissue. This needs to be performed in a large animal model that is better representative for humans, preferably in preclinical models using dysfunctional/diseased bladders.

Acknowledgements

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in de delta' (PID 101020). We would like to thank the employees of the Central Animal Laboratory, Radboud university medical center, for their outstanding assistance at the performed animal operations.

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Antigen	*Antibody*	*Source*	*Dilution*	*Antigen retrieval*
Cytokeratin 7	RCK105	MUbio BV	1:10	A
Pancytokeratin AE1/AE3	AE1/AE3	Thermo Fisher scientific	1:800	B
Vimentin	V-9	BioGenex	1:2000	C
α-smooth muscle actin	1A4	Sigma-Aldrich	1:15000	C
Desmin	[33]	BioGenex	1:200	C
Smoothelin	R4A	Santa Cruz Biotechnology	1:150	A
S100	Z0311	Dako	1:10000	A

Table 1. Antibodies used for immunohistochemical evaluation. **A**: Heat mediated in sodium citrate buffer (10 mM; pH 6.0; 10 min; 100°C); **B**: with 0.1% pronase (30 min at room temperature); **C**: without antigen retrieval.

Animal	*Animal weight (kg)*					*Bladder capacity (ml)*					*Regenerated area (cm²)*			
COL	Preop	Mean	3 m	Mean	In-crease	Preop	Mean	3 m	Mean	In-crease	Area	Mean	SD	Con-traction
1	43		90			NA*		850			10.9			
2	47	49.7	108	105.3	112%	460	580	1063	1268	150%	6.6	8.3	2.3	61.0%
3	59		118			700		1890			7.3			
COLHEP														
1	44		103.5			880		NA*			12.1			
2	46	44.7	115	109.2	144%	770	718	NA*	1400	177%	11.4	14.0	3.9	34.0%
3	44		109			505		1400			18.5			
COLHEPGF														
1	41		Died			650		Died			NA			
2	50	49.5	120	115	132%	720	611	1238	1456	155%	9.6	10.6	1.5	49.8%
3	53		NA+			580		NA+			NA			
4	54		110			495		1675			11.7			
Sham														
1	46		94			350		915						
2	47	49.7	115	104.5	110%	350	383	1300	1137	199%				
3	56		NA			450		1195						

Table 2. Overview of characteristics before surgery and at the evaluation time point after 3 months. No significant differences in weight existed within and between groups. No statistically significant differences in bladder capacity were found between groups. The largest amount of tissue was regenerated in the COLHEP-group, which was statistically significant compared to the COL-group (p=0.002) (also see Fig. 4a), but not compared to the COLHEPGF-group (p=0.083). Several values are not available due to drop-out before 3 months or technical failure, NA = not available, * = catheterization impossible, + = sacrificed. Preop = pre-operative; 3 m = 3 months after operation; SD = standard deviation.

	COL-group	*COLHEP-group*	*COLHEPGF-group*
Urothelium	++	++	++
Angiogenesis	++	++	++
Vimentin-positive cells	+/-	+	+/-
α-SMA-positive cells	+	++	+
SMCs	+	+	+
ECM	+/-	+/-	+/-
Inflammation	+	+/-	+/-
Scaffold remnants	Sp	+/-	Sp
Elastic fibers	Sp	+/-	Sp
Nerves	Sp	Sp	Sp

Table 3. Overview of microscopic scoring of histological results. COLHEP showed more vimentin and α-SMA positive cells than COL or COLHEPGF as well as more scaffold remnants and elastic fibers. ECM was also slightly more visible in COLHEP, although not visible in table due to round off in categories. More inflammation was seen in COL compared to COLHEP and COLHEPGF. No other differences were found. ++ = abundant, + = present, +/- = limited, Sp = sporadically present.

Chapter 7

Ureteral reconstruction with reinforced collagen scaffolds in a porcine model

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Abstract

Repair of long ureteral defects often requires extensive surgical procedures and graft tissue which is associated with various complications, e.g. graft site morbidity or lack of suitable tissue. Tissue engineering might provide an alternative therapeutic approach in these cases. Reinforced tubular collagen-Vicryl scaffolds (l = 5 cm, lumen Ø = 6 mm, thickness = 3 mm) were developed with or without vascular endothelial growth factor and basic fibroblast growth factor and evaluated in a pre-clinical porcine model. The reinforced scaffolds were successfully implanted in 20 pigs and functional, macroscopic and microscopic evaluation was performed at one and three months. Two animals without scaffold implantation served as control. All animals survived until their predetermined evaluation time point and grafted scaffolds showed urothelial regeneration, smooth muscle cell ingrowth and neovascularization. Loopogram and macroscopic evaluation revealed constriction of the scaffold lumen and hydroureteronephrosis. Enhanced muscle ingrowth was observed in growth factor loaded scaffolds, but this was not significant. We conclude that reinforced collagen-Vicryl scaffolds are mechanically suitable for ureteral repair, but further optimization to prevent strictures is required.

Introduction

Ureteral traumas account for approximately 3% of all urogenital traumas and are mainly of iatrogenic cause. Of these, gynaecological surgeries account for about 73% of all ureteral injuries, mainly during hysterectomy [10-12]. When recognized during surgery, primary repair is often possible. However, in 33-88% of the cases injury is not recognized in time, potentially leading to urinoma, sepsis and even kidney loss [13, 14]. The repair of long defects, where end-to-end anastomosis is not feasible, is often challenging for the surgeon. Depending on the type and location of the injury, different procedures such as a Boari flap, transureteroureterostomy, and ileal interposition are used [15, 16]. A shortage of suitable donor tissue can complicate the repair. Therefore, various biomaterials have been studied as an alternative to the current techniques in the past three decades, but high failure rates were observed [17].

In a previous study we evaluated the regenerative capacity of collagen based tubular scaffolds in the reconstruction of the porcine ureter. The lack of mechanical strength complicated the surgical procedure and regeneration outcome [18]. To reinforce the tubular scaffolds, a Vicryl mesh was introduced in the collagen to generate a reinforced construct, which was then loaded with growth factors (vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)) to improve the cellular ingrowth. The aim of this study was to investigate the regenerative outcome of ureteral repair after introduction of a large defect, using reinforced scaffolds in a porcine model.

Materials and Methods

Reinforced scaffold production

Type I collagen was purified from bovine Achilles' tendons using a previously described protocol (Figure 1)[19]. Commercially available Vicryl meshes (Ethicon) were tubularized using Vicryl sutures (Ø = 8 mm). Tubular reinforced 0.5% (w/v) collagen-Vicryl constructs (l = 5 cm, lumen Ø = 6 mm, wall = 3 mm) were prepared by freezing homogenized collagen with the Vicryl mesh in a cylindrical mold, followed by freeze-drying [20]. Carbodiimide cross-linking was used to cross-link the constructs, with or without 0.25% (w/v) heparin (Diosynth). Constructs were packaged in blisters containing 70% (v/v) ethanol and were sterilized by 25 kGy γ-irradiation (Synergy Health). A 2,4,6-trinitrobenzene sulfonic acid assay and hexosamine assay was used to determine the degree of collagen cross-linking and to quantify the amount of bound heparin, respectively. Ultimate tensile strength of collagen was analyzed by elongating five wet collagen strips (10 x 30 mm) and four Vicryl meshes (10 x 30 mm) at 2 mm per second using a Biodynamic™ bioreactor (Bose). The ultra-structure of the scaffold was characterized by scanning electron microscopy (Figure 1d).

Growth factor loading

One hour before surgery, the heparin loaded scaffolds were incubated with 0.5 µg/ml recombinant human VEGF-165 and 0.5 µg/ml recombinant human bFGF

(both R&D systems) in 3 ml PBS. The scaffolds were washed 3 times with PBS for approximately 15 min before implantation. Western blot analysis was used to quantify the concentration of loaded growth factors [21].

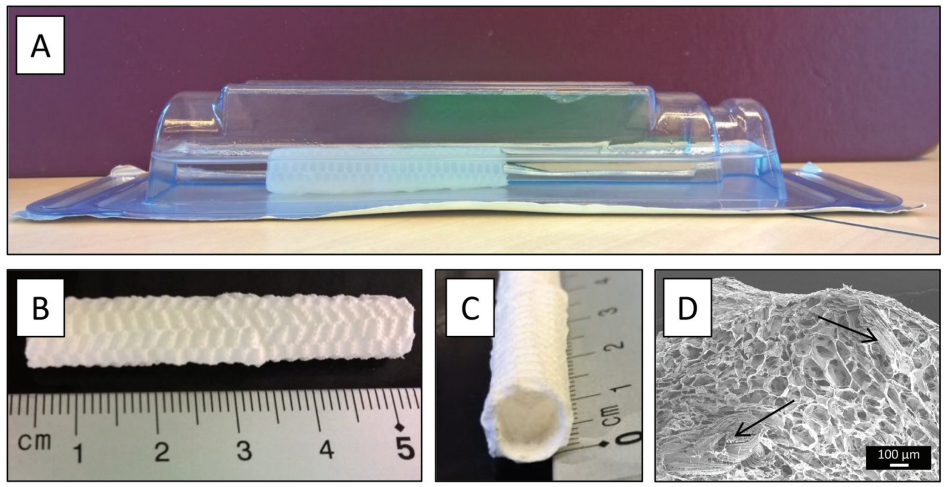

Figure 1. A. Wrapped reinforced scaffold in 70% ethanol. **B**. Macroscopic view of the reinforced scaffold. **C.** The reinforced scaffolds retain an open lumen under their own weight. **D**. Scanning electron microscopy (SEM) of the cross section of a reinforced scaffold. A highly porous structure is visible with good integration of the collagen and the Vicryl (black arrows). 50x magnification.

Animals

All procedures were performed according to the Institute of Laboratory Animal Research guide for Laboratory Animals [22]. The Nijmegen Medical Center animal ethics committee approved the study protocol (RU-DEC-2012-320). In total, 22 female Landrace pigs weighing around 50 kg were used. They were housed in groups at the Institute's farm with a restricted diet and free access to water. The pigs arrived at the animal laboratory at least 24h before every procedure. The pigs were weighed before surgery and were pre-medicated by intramuscular injection of 10 mg/kg ketamine (Eurovet Animal Health), 1 mg/kg midazolam (Roche) and 50 µg/kg atropine sulfate (Pharmachemie). All pigs were subjected to a scaffold implantation (n=20) or primary anastomosis of the ureter (n=2), and were evaluated one (n=12) or three months (n=10) post-implantation.

Pre-operative preparation

Intravenous fluids (lactated Ringer's solution) and anesthesia (2.5 mg/kg propofol (B.Braun)) were administered through the auricular vein. Pigs were intubated and 2 L/min O2/N2O (2:1) containing 0.5% isoflurane (Baxter), combined with a bolus of 0.3 mg/kg midazolam (Roche) and 5 µg/kg sufentanyl citrate (Janssen-Cilag) was administered. Anesthesia was maintained with 0.6 mg/kg/h midazolam (Roche), 10 µg/kg/h sufentanyl citrate, 0.2 mg/kg Vecuronium bromide (Organon) and 25 mg/kg atropine sulfate. Pigs were placed on a heating pad and covered with a sterile surgical blanket after the surgical area was shaved, washed and disinfected using iodine. Electrocardiography, pulse oximetry, CO_2 and temperature registration provided continuous monitoring during surgery. Before surgery, the bladder was catheterized (12 Ch Nelaton catheter).

Surgical procedure

A 10 cm right flank incision was made 5 cm below the spine and parallel to the lower rib. The retroperitoneal space was entered after division of the muscle layers. Subsequently, the ureter was located and mobilized. Approximately 5 cm of the ureter was removed and the ureteral ends were spatulated. Reinforced scaffolds, with or without growth factors, were placed over a 7 FR/22-32 cm multi-length double J ureteral stent (Inlay Optima, Bard medical), which was then positioned in the kidney and urinary bladder. Using interrupted 6-0 monocryl (Ethicon) sutures, a tension free anastomosis was created. The anastomosis sites and the scaffold were then covered with fibrin glue (Tisseel, Baxter). Approximately 1 cm from the anastomosis site, two 6-0 prolene sutures were placed on either side as reference. In two control animals, the ureter ends were spatulated and anastomosed without placing a scaffold. Finally, the fascia (0 Vicryl), 4 muscle layers (2-0 Vicryl), subcutis (2-0 Vicryl) and skin (1 CT) were closed (all sutures from Ethicon). The animals were followed closely for signs of discomfort and leakage from the anastomosis.

Neo-ureter evaluation

One and three months after the implantation procedure, the animals were sacrificed by an overdose of intravenous pentobarbital after sedation with midazolam/ketamine (Roche, Eurovet Animal Health) and evaluated radiologically. Iodinated contrast fluid in a 1:1 dilution with saline (Aguettant) was injected into the pyelum followed by a loopogram to evaluate lumen size and accessibility. The urinary tract was harvested and evaluated macroscopically. Specimens of the neo-ureter, ureters and the kidneys were harvested and fixed in 4% (v/v) formaldehyde in PBS (Boom) overnight for evaluation.

Immunohistochemistry

All tissues were embedded in paraffin, cut and stained with hematoxylin (Klinipath) and eosin (Merck). Additionally, immunohistochemistry for Pancytokeratin (Fisher Scientific), Smoothelin (Santa Cruz Biotechnology), desmin (Biogenix), α-smooth muscle actin (αSMA, Biogenix) and vimentin (Fisher Scientific), and Masson's Trichrome was performed on transverse sections of the neo-ureter. For immunohistochemistry, sections were blocked in 1% (v/v) H_2O_2 (Merck) in PBS. Antigen retrieval for Pancytokeratin and Smoothelin was performed using microwave treatment with sodium citrate (pH 6). Sections were then incubated with 5% (v/v) goat serum (Bodinco) for 10 min. Primary antibodies (pancytokeratin (1:800), desmin (1:400), vimentin (1:1 000), αSMA (1:8 000) and Smoothelin (1:75)) were incubated for 1 h. Secondary antibody (Poly-Horseradish peroxidase-goat-anti-mouse/rat/rabbit IgG, Immunologic) was pre-incubated with 10% (v/v) swine serum (prepared in house) for 30 min, followed by an additional 30 min incubation

on the slides. Finally, 3,3'-Diaminobenzidine (bright-DAB, Immunologic) was used to develop the slides and sections were counterstained with hematoxylin for 5 s. All antibodies and goat serum were diluted in PBS with 1% (v/v) Bovine serum albumin (BSA, Sigma-Aldrich).

Transverse neo-ureter sections and affected kidneys were scored for hydronephrosis, neo-ureter development and immune response by two independent evaluators (PdJ, PG). In addition to independent scoring of the slides, quantitative analysis was performed on desmin stained neo-ureter slides to quantify smooth muscle tissue ingrowth. Briefly, using ImageJ, desmin stained slides were corrected for background using a 'rolling ball method' (50 px) and converted to binary. Next, the pixel intensity along the native muscle and neo-ureter was plotted. This data represents the amount of smooth muscle ingrowth from the native ureter tissue into the neo-ureter.

Results

Scaffold characterization

The tubular scaffolds were highly porous and the Vicryl mesh was fully integrated into the collagen scaffold (). The collagen was stabilized by EDC/NHS cross-linking, which resulted in a 49% (no heparin) and 61% (with heparin) free amine group reduction. Incorporating the Vicryl mesh greatly improved handling properties: the lumen stayed open and both ends could easily be sutured. The tensile strength of the Vicryl meshes was around 20x stronger than the cross-linked collagen structure (collagen: 1.46 ± 0.41 N, Vicryl: 29.78 ± 8.41 N). The heparinized scaffolds contained 20 wt% of heparin. Western blot analysis showed that an average of 10.0 ng VEGF/ mg collagen and 50.3 ng bFGF/ mg collagen were bound per scaffold at the time of implantation. Each scaffold contained approximately 35 mg of collagen, therefore we estimated that a total of 0.35 µg VEGF and 1.76 µg BFGF was delivered at the implantation site.

In vivo evaluation

Figure 2 shows an overview of the implantation procedure. All animals survived until the pre-determined time point (one or three months). In both control animals urinary leakage was observed due to inadequate healing of the end-to-end anastomosis upon evaluation at one month. Both control animals could not be further evaluated due to the extent of the leakage. In three animals a urinoma was observed after one month (n = 1 with growth factors, n = 2 without growth factors). One additional animal grafted with a scaffold without growth factors presented with a small encapsulated urinoma after three months. The other animals did not show any urine leakage, diverticula or fistula formation. All grafted animals developed constriction of the neo-ureter, including the anastomoses. The double-J stent remained *in situ* during the entire experiment, but the proximal part of the stent migrated from the kidney to the proximal ureter, possibly due to dilatation of the proximal ureter. The ureters were open even when the construct constricted. In all cases, the loopograms revealed a lumen which matched the size of the double-J stent (Figure 3c). One month post-grafting of the growth factor loaded scaffolds the lumen of the regenerating tissue had a red appearance, but this was not apparent three months post-grafting (figure 3d-g). All kidneys of the anastomosed ureters showed signs of hydronephrosis as evidenced by a whitish appearance, distension and dilatation of the kidney.

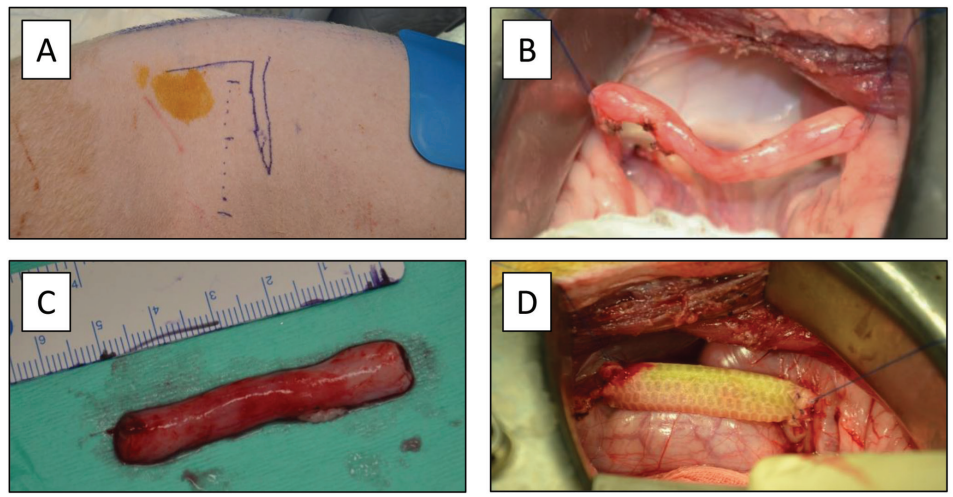

Figure 2. Overview of the surgical procedure. **A.** A flank incision next to the lower rib was used to approach the ureter. **B**. The ureter was localized and mobilized. **C**. Approximately 5 cm of the ureter was removed. **D.** A reinforced scaffold was placed over a 7 Fr. Double-J stent and sutured to the spatulated ends of the ureter.

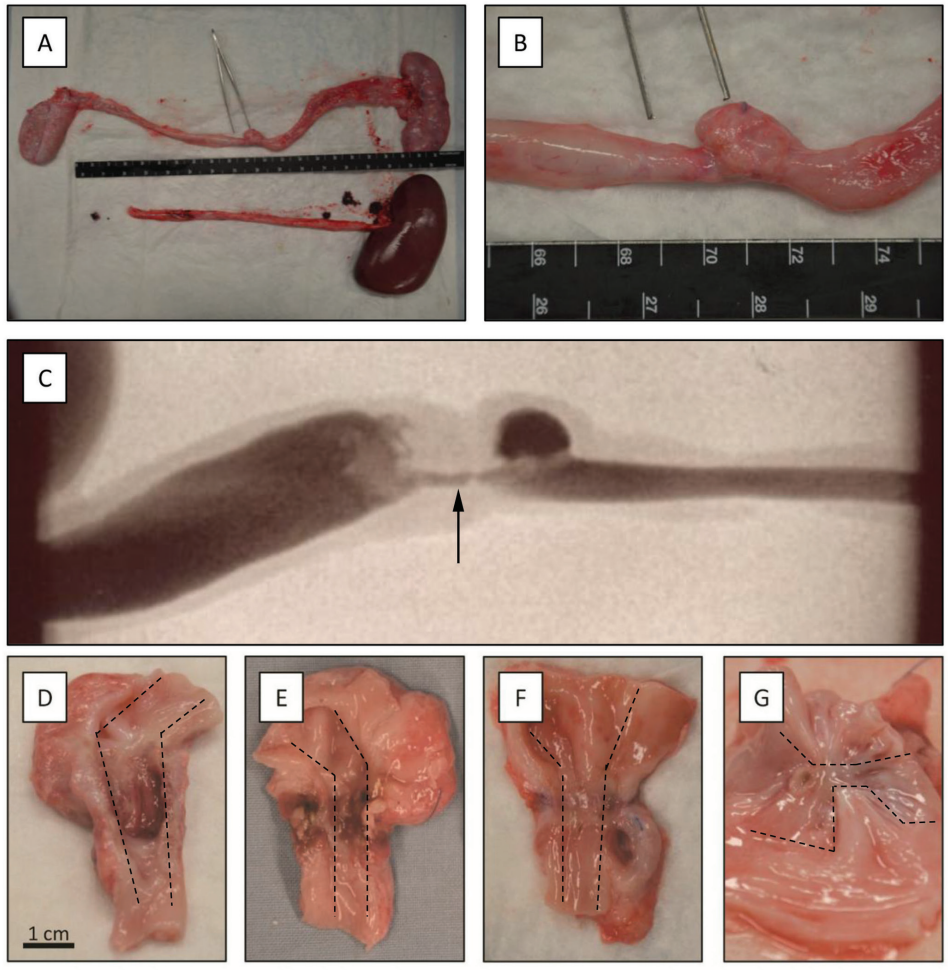

Figure 3. A. The urinary tract was harvested at the evaluation time point. A dilated ureter and hydronephrotic kidney was observed. B. Implantation location shows signs of tissue contraction and a small diverticulum. C. Loopogram three months after implantation of a scaffold without growth factors. Left: Proximal ureter. Right: Distal ureter. Arrow indicates the implantation site. The proximal ureter shows dilatation, while the distal ureter has a normal diameter. The implantation site shows narrowing of the lumen, leaving a negative imprint of the double-J stent. D-G. Macroscopic view of implantation area after dissection. Dotted lines indicate the lumen. D. One month, growth factor loaded. E. One month, no growth factors. F. Three months, growth factor loaded. G. Three months, no growth factors.

Histology

Microscopic scoring was performed by two independent researchers (PdJ, PG, Table 1), focusing on the appearance of the urothelium and submucosa layers of the neo-ureteral tissue and the affected kidney. Figure 4 shows representative samples of the different groups.

		1M		*3M*	
		-GF	*+GF*	*-GF*	*+GF*
Urothelium	Hyperplasia	+/-	+/-	sp	-
	Squamous	-	sp	sp	sp
	Intact	+/-	+/-	++	++
	Umbrella cells	-	+/-	sp	sp
	Cell layers	+	+	+	+
	Basal layer	+/-	+	+/-	+
	Granulocytes	sp	+/-	+/-	+/-
	Matrix present	-	-	sp	sp
Submucosa	ECM organization	+	+	++	++
	Neovascularization	+	+	+	+
	Muscle ingrowth	+/-	+/-	+	+*
	Myofibroblasts			+	+
	Scaffold remnants	+/-	+	+/-	+/-
	Granulocytes	+/-	+/-	+/-	+/-
	Lymphocytes	++	++	+	++
	Macrophages	+/-	+/-	+/-	+/-
	Giant cells	+	+/-	+/-	+/-
Kidney	Hydronephrosis	+	+/-	++	+++

Table 1. Microscopic evaluation by two independent researchers (PdJ, PG). Urothelium was evaluated for being continuous, different cell types and possible abnormal cell growth. The submucosa was evaluated for organization of the tissue, including extracellular matrix organization, neovascularization and muscle ingrowth. Immune response was also studied. Finally, hydronephrosis of the kidney was evaluated. Scores between researchers were averaged. Scoring possibilities: - (not present), sp (sporadically present), +- (somewhat present), + (present), ++ (abundant), +++ (very abundant). +* indicates a higher scores than +, but lower than ++.

One month

By one month neo-ureters were intact with signs of regeneration. The control animals could not be analyzed due to extensive urine leakage.

Urothelium

A single layered urothelium was formed throughout most of the lumen of all neo-ureters as judged by the presence of a pancytokeratin-positive cell layer regardless of the addition of growth factors. However, the urethral basal cell layer was more identifiable and occasionally umbrella cells were observed when growth factor loaded scaffolds were used. Sporadically squamous differentiation as well as more granulocytes were observed in the remodeled growth factor loaded scaffolds, albeit that the number of granulocytes was still rare.

Submucosa

The extracellular matrix tended to align the lumen of the neo-ureter, irrespective of growth factor loading. Neo-vascularization was observed as well as the initiation of smooth muscle tissue ingrowth into the neo-ureter, but no differences were found between the groups. More collagen remnants were found in the growth

factor loaded scaffolds. Vicryl remnants were absent, and the immune response was similar in all animals, mainly consisting of lymphocytes and a limited influx of granulocytes and macrophages. Interestingly, more giant cells were observed in the group without growth factors, even though less collagen remnants were observed.

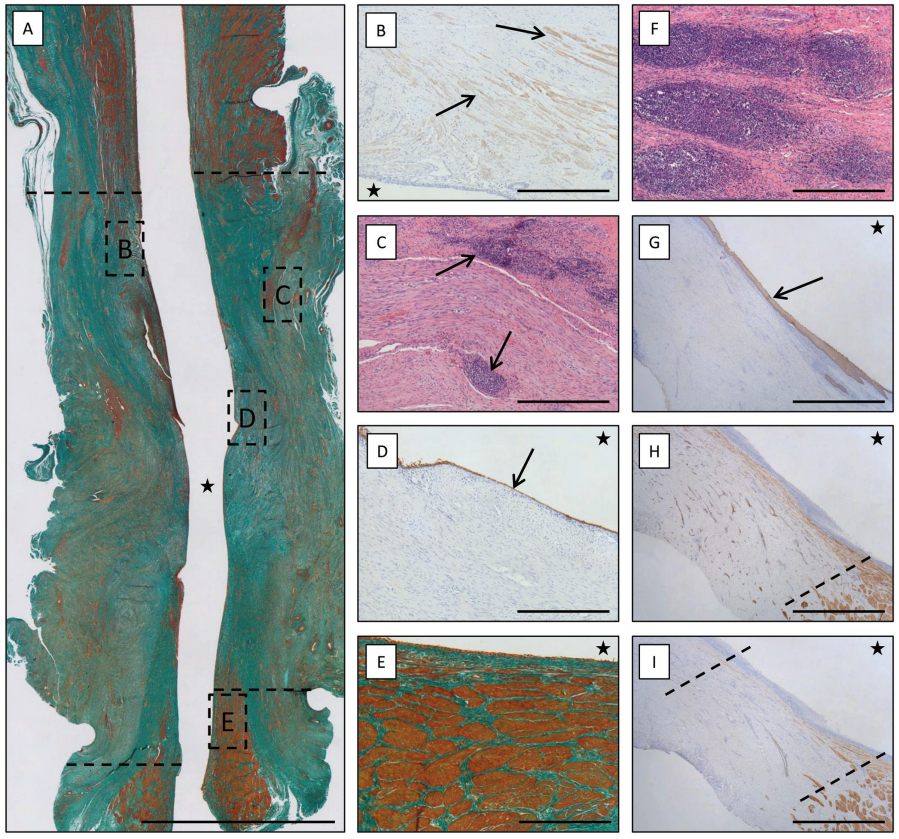

Figure 4. Histological overview of the regenerated ureter. **A**. Masson Trichrome stained example of a longitudinal section across the native ureter. Scalebar = 5mm **B**. Smoothelin staining at the anastomosis indicating development of new muscle. **C**. A moderate immune response with mainly lymphocytes. **D**. Pancytokeratin staining in the middle of the new tissue, indicating formation of urothelium. **E**. Native smooth muscle tissue, clearly showing normal muscle architecture. **F**. Immune response at one month. Mainly lymphocytes and some giant cells were found. By three months, this response became much weaker (C) **G-I.** Anastomosis at three months with growth factors, stained for pancytokeratin staining (G), desmin (H) and smoothelin (I). The luminal side was covered with a (multilayered) urothelium. Desmin positive cells were found further into the new tissue than smoothelin, indicating the formation of new and maturing muscles in the new tissue. Scalebars B-I = 500µm. * Indicates the lumen.

Kidney

All kidneys showed signs of hydronephrosis, although the kidneys connected to ureters with growth factor loaded constructs showed more intact glomeruli, tubuli and less fibrosis.

Three months

By three months the remodeled scaffold started showing tissue organization, as evidenced by a well-organized extracellular matrix, growing muscle layer and urothelial lining.

Urothelium

The neo-ureter was completely covered by at least one single layer of cytokeratin-positive cells. The basal cell layer was more clearly identifiable in the growth factor loaded group, but no difference was seen in the amount of umbrella cells. Also, the number of granulocytes and squamous cells was similar. Scaffold remnants were sporadically observed in both groups.

Submucosa

In both groups the extracellular matrix was well organized in parallel with the lumen. Myofibroblasts were abundant in the neo-ureter, whereas the amount of neo-vascularization and scaffold remnants and the number of giants cells did not change in comparison to 1 month post-implantation. The inflammatory response appeared to be lower in the group treated without growth factors, as evidenced by reduced lymphocyte infiltration. Granulocyte and macrophage infiltration remained unchanged. More importantly, the smooth muscle layer had continued to develop and was growing into the regenerating new tissue as thin, stretched bundles. These smooth muscle bundles appeared more evident in the remodeled growth factor loaded scaffolds.

Kidney

In contrast to one month post-grafting, hydronephrosis was more pronounced in all animals. Kidneys harvested from animals grafted with scaffolds without growth factors sporadically showed regions with intact tubuli and glomeruli, whereas with growth factor most of the normal morphology had disappeared.

Quantification of muscle ingrowth

Desmin stained slides were used to quantify the total ingrowth of the smooth muscle cells in the neo-ureter (figure 5). The mean length of the neo-ureter for the non growth factor loaded scaffolds and growth factor loaded scaffolds was 11.9 ± 5.3 mm and 11.3 ± 4.6 mm respectively by three months. Average smooth muscle cell ingrowth was 2.0 ± 1.7 mm without growth factors and 2.4 ± 1.0 mm with growth factors from both sides. Relative to the total defect size, the ingrowth of muscle tissue was 32 ± 15 % without growth factors and 50 ± 34 % with growth factors.

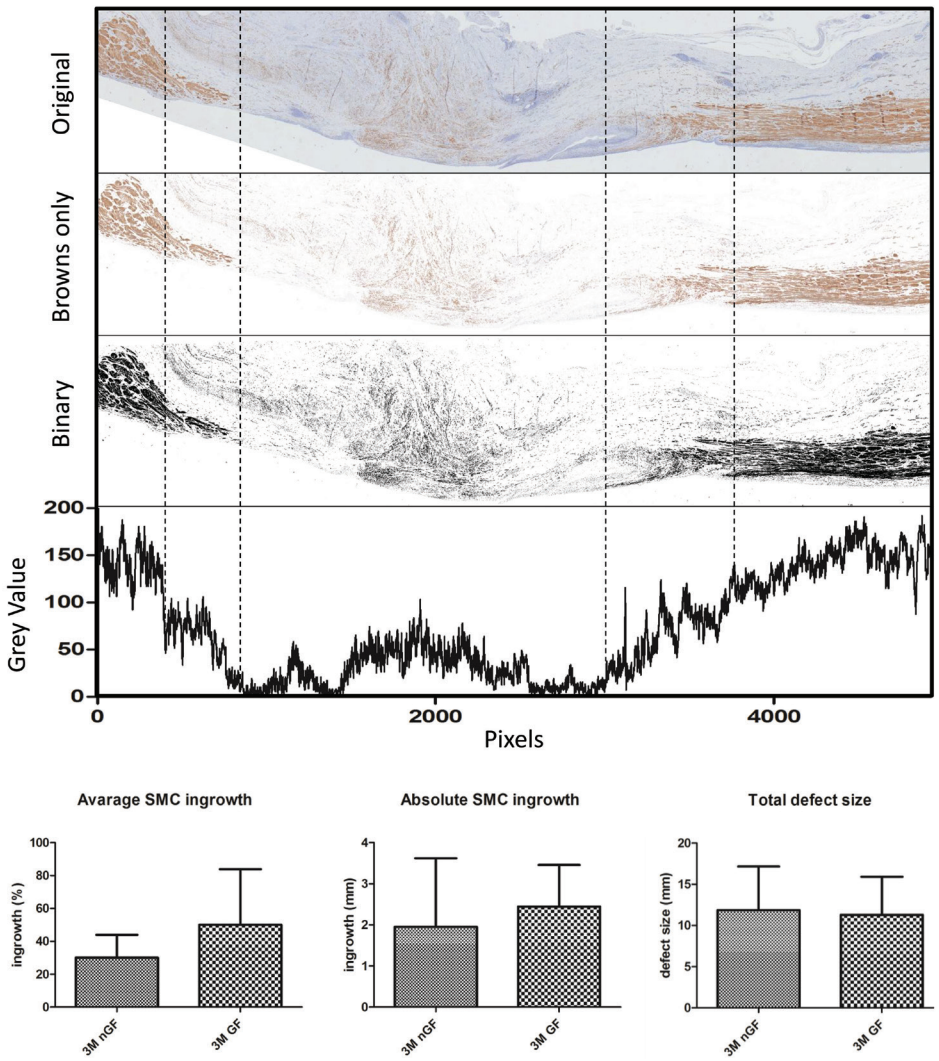

Figure 5. Quantitative analysis. An example is shown for a sample from the three month group without growth factors. After background correction of the original image, the browns (desmin positive tissue) were extracted from the image. Next, the image was converted to binary. Finally, a profile plot of the pixel intensity along the construct was prepared. Intensity increases with increased desmin staining on the slide. The plots of all samples were used to quantitatively determine the total defect size and ingrowth of smooth muscle tissue from both sides (dotted lines). No significant influence on the use of growth factors was found.

Discussion

Compared to tissue engineering of the urethra, urinary bladder and urinary diversions, the ureter is relatively understudied. This might be attributed to a lower number of lesions, a positive-results bias or a lack of cross-over from other studies in which tissue engineered tubular organs were evaluated [23]. In addition, while several studies on tubular scaffolds were published, they were not specifically designed or evaluated for ureter repair or only for onlay repair. Nevertheless, there is a need for tissue engineered constructs when large defects need to be repaired.

The reinforced tubular scaffolds with predetermined mechanical properties, consisting of collagen and Vicryl mesh, with and without growth factors, were successfully anastomosed in pig ureters, creating a watertight and tension free neo-ureter. The introduction of a Vicryl mesh increased the mechanical strength of the tubular constructs up to 24 times greatly improving the surgical handling. More importantly, no early complications were seen in the animals as a result of inadequate stability, in contrast to tubular grafts that consisted of collagen only [18]. Consequently, all animals survived until the predetermined evaluation time points without major short-term complications.

Already after one month the Vicryl woven mesh was completely degraded without a major inflammatory response. This is in accordance with a recent study in which different polymers were tested for bladder regeneration and the collagen-Vicryl construct turned out to be the most suitable candidate construct for urogenital tissue engineering [24]. Although the clinical outcome of the scaffold grafting was favorable, the tubular scaffolds were created by manually tubularizing commercially available Vicryl meshes, which is not truly compatible with clinical practice. Therefore, Vicryl tubes should be produced by e.g. electrospinning, knitting and winding to achieve clinical implementation by high throughput production. This will also allow further fine-tuning of the construct properties.

To enhance the biological characteristics of the construct growth factors VEGF and bFGF were included to stimulate cell ingrowth from the adjacent native ureters and tissues [25]. The ingrowth of smooth muscle tissue and the formation of a urothelial barrier are the most important outcome measure as it plays a critical role in the peristalsis of the ureter and preventing tissue damage. Considering that a fully regenerated musculature is essential for the autonomous ureteral peristalsis, the presence of smooth muscle cells was quantified using image analysis of desmin stained slides. The objective image analysis allowed accurate determination of the total size of the remodeled implanted construct and the distance of smooth muscle ingrowth, both good indicators of construct contraction and muscle development in the newly formed tissue. The results indicated a trend towards increased muscle ingrowth when growth factors were added, however, the effect was not significant. Regarding the urothelium, we observed the formation of a partial and complete single layered urothelium at one month and three months respectively, regardless of growth factor loading. Interestingly, there was extensive graft shrinkage from 50 mm (implanted scaffold) to 11.9 ± 5.3 mm (no growth factors) and 11.3 ± 4.6 mm (growth factors), which may have played a role in the formation of hydronephrosis. Even though VEGF and bFGF should increase angiogenesis in acellular collagen scaffolds [26], our results did not reveal major differences between growth factor loaded and non growth factor loaded constructs even though the growth factor concentrations were supranatural: They were in the ng/mg range, compared to natural pg/

mg range [27]. The lack of effect may be attributed to the delivery method. We used heparin sulfate to concentrate the heparin-binding growth factors bFGF and VEGF in the construct. Although heparin enabled effective loading of the scaffolds, the binding affinity may have been too low. The immediate urinary flow after implantation may have caused an unfavorable fast release. It is therefore likely that the growth factor exposure was limited and probably only effective during early stage wound healing. In order to accomplish sustained growth factor delivery, alternative delivery methods should be considered. These could include covalent binding to the collagen, hydrogels such as fibrin and polymeric carriers that protect the growth factors from the urinary flow [28-30].

To assess the functional outcome of the neo-ureters we investigated the kidneys at all evaluation points. Hydroureteronephrosis was observed in all pigs as a result of contraction of the inner lumen of the construct. This led to urine retention in the upper urinary tract probably creating increased pressure in the kidney and proximal ureter. Three possible underlying causes may include: (i) A lack of peristaltic movement in the large scaffold after implantation. This may have prevented adequate urine transport past the regeneration area. Restoring the peristaltic activity may be key in prevention of urine retention in the upper urinary tract and regeneration of the muscular layers should be prioritized when attempting to regenerate the ureter. (ii) A potential tension on the anastomosis due to retraction of the ureter and contraction during wound healing. Replacing a certain sized defect by a longer scaffold may prevent this. (iii) Despite the favorable anatomy, the pig has unavoidable drawbacks in relation to this application; e.g. high growth rate, quadrupedal stance, high intra-abdominal pressures, and rapid wound healing with extensive fibrosis [31, 32]. The wound healing and contraction may have caused strictures around the double-J stent, disabling the urinary flow which ultimately resulted in hydroureteronephrosis. Alternatives to the pig model might include goats, sheep and dogs. These animals are more mobile than pigs, which may improve urine drainage. Also, their wound healing shows less fibrosis as supported by the fact that a stoma can be maintained e.g. in goats, but not in pigs [33, 34]. However, there will always be dissimilarities between the clinical and pre-clinical situation, e.g. local drain or a nephrostomy catheter could be placed more easily in the clinical situation to drain a urinoma or prevent large amounts of urine from passing the anastomosis site.

The inflammatory response appeared to be more profound (chronic) when scaffolds were loaded with growth factors as indicated by a higher number of lymphocytes three months post-grafting. Additionally, scaffold remnants were observed. This difference in scaffold clearance and resulting inflammatory response may have been caused by the heparin sulfate. After the growth factors were washed out, the heparin may have protected the collagen from proteolysis due to its high negative charge delaying matrix degradation [35]. In agreement with our previous results, the presence of multinucleated giant cells indicated a foreign body response against the scaffold. This was probably due to the relatively high degree of cross-linking (49% (no heparin), 61% (heparin)) and the inclusion of the synthetic polymer [36, 37].

Until recently, the field of ureteral tissue engineering was dominated by tubular materials such as small intestinal submucosa, collagen, and Gore-Tex without cells. There is a trend towards the use of more smart and biodegradable materials with or without mesenchymal stem cells and/or pre-implantation techniques. The regenerative outcome of the tubular mechanically enhanced scaffolds studied here was comparable with previous studies in which collagen sponges and SIS patches were used [23]. Our results were superior to studies using Gore-Tex or acellular matrix, which did not induce cellular ingrowth [38, 39]. Inclusion of mesenchymal and adipose-derived stem cells seeded on a mechanically suitable degradable material has shown promising results for ureteral tissue engineering [40, 41]. Relatively large defects (> 3cm) were repaired in rabbits, which supports the current view that cellular constructs are required when repairing defects that warrant more than one cm tissue [42]. By incorporating these stem cells, strictures were avoided and smooth muscle cell and urothelial cell regeneration was greatly improved.

Another potential method to reduce tissue contraction and thereby hydronephrosis may be to pre-implant the constructs in a different, highly vascularized tissue. This way the initial tissue contraction takes place in a more controlled environment, without the toxic influences of urine [43]. Recent studies have shown the successful application of these techniques by implanting the tissue in non-functional locations [44-46], as well as using them for ureteral repair afterwards [41, 47]. Pre-implantation before functional implantation may therefore improve the outcome of the reconstruction.

Conclusions

Reinforced collagen-Vicryl scaffolds might be a suitable base material for ureteral repair and may provide an alternative to current graft tissue and reconstructive surgeries in the future. We observed both urothelial lining and smooth muscle cell ingrowth with an average of 32% and 50% of the total neo-ureter by three months, which indicates the potential of the material. However, a more appropriate animal model should be chosen to more adequately mimic the human situation, and a more suitable method for sustained delivery of growth factors in case of ureteral repair should be explored.

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Chapter 8

Clinical protocol levels are required in laboratory animal surgery when using medical devices: Experiences with ureteral replacement surgery in goats

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Abstract

In large animal studies it is common to test medical devices that are or could also be used in patients. In this short report we describe the use of a ureteral J-stent for the evaluation of biodegradable tubular constructs for tissue reconstruction and the regeneration of the ureter in Saanen goats. Similar to a previous study in pigs, the ureteral J-stent was blindly inserted until some resistance was met. During evaluation of the goats after three months, we observed perforation of the renal cortex by the stent in four out of seven animals. These results indicated that blind stent placement was not possible in goats. In four new goats, clinical protocols were followed using X-ray and iodinated contrast fluids to visualize the kidney and stent during stent placement. With this adaptation the stents were successfully placed in the kidneys of all four goats with minimal additional effort. It is likely that other groups in other fields ran into similar problems that could have been avoided by following clinical protocols. Therefore, we want to stress the importance of following clinical protocols for the use of medical devices in animals to prevent unnecessary suffering and to reduce the number of animals needed.

The application of new surgical techniques often requires evaluation in large laboratory animals to study its effectiveness and safety [1]. This is especially important in emerging fields such as tissue engineering and regenerative medicine. Tissue engineering aims to replace damaged organs and tissues by providing an alternative to autologous donor tissue or when standard surgical procedures are not possible [2]. One tissue engineering application that we are interested in, is replacing a long segment of the ureter with a tubular template after trauma or iatrogenic injury [3]. To prevent obstruction and kidney damage a ureteral stent is placed during surgery. This stent facilitates sufficient urine transport during the initial wound healing phase, which is accompanied by tissue contraction and scar formation. A "J" tail curl at the ends helps to keep the stent in the correct place [4]. In previous pig studies by our group, blind insertion of this stent into the proximal ureter/pyelum of the kidney was uneventful [5].

Recently, we have switched from young Landrace pigs to adult goats as our animal model for ureter reconstruction as the pigs were growing too fast (30 kg weight gain in 3 months). The rapid growth was also associated with fast wound healing as evidenced by fast skin closure in a stoma model, which may have influenced the regenerative outcome [6]. Despite the smaller overall size of the kidneys, pyelum and ureteral diameter, stenting was easily performed in cadaver material in preparation for the animal experiment.

Initially, seven female Saanen goats weighing 50-70 kg were used in this study (based on power analysis). The study was approved by the Nijmegen Medical Center animal ethics committee (RU-DEC-2014-223) and all procedures were performed according to the Institute of Laboratory Animal Research guide for Laboratory Animals [7]. As catheterization was required, only female goats were used due to the curved urethra in male goats. The goats were housed in groups at the Institute's farm with free access to water and a restricted diet. The goats were sedated per institute's protocol prior to surgery and treated with analgesia and antibiotics afterwards. In all goats, the right ureter was located and mobilized through a flank incision. The ureter was cut, spatulated and the ureteral stent (4.7Fr/22-32 cm multi-length double J stent, Inlay Optima, Bard medical) was blindly inserted into the proximal ureter until some resistance was met as indicator for correct positioning. At that point, the guide-wire was removed to allow the proximal "J" to curl inside the pyelum. The tissue engineered template was positioned over the stent and the stent was then inserted into the distal ureter and urinary bladder. Finally, the tissue engineered template was connected to both spatulated openings of the ureter by end-to-end anastomosis.

After three months the goats were sacrificed by an overdose of pentobarbital for evaluation. We noticed that the stents had perforated the kidney in 4/7 treated goats (Figure 1). In one case this resulted in a large urinoma on top of the kidney, while in the other three cases inflammation in the kidneys was noted. This clearly is an unwanted result; the wound healing results are unreliable and it may have caused unnecessary suffering for the goats. Upon close inspection of the harvested tissue, it was obvious that the kidney tissue of the goats is much softer than that of pigs. This may have been missed in the cadaver study due to the room

temperature and the clotting of blood in the highly vascularized kidney, which may have increased the stiffness and resistance of the kidney. In addition, due to the small size of the renal calyces and pyelum, it was difficult for the "J" end of the stent to correctly curl inside the kidney. Therefore, it is more likely that the stent penetrated the kidney cortex during placement.

In routine human clinical practice, ureteral stents are almost always inserted using contrast fluids and X-ray guidance [7]. This allows visualization of the kidney and correct positioning of the stent inside the pyelum can be verified. In view of the observed kidney perforations we used iodinated contrast fluids (Xenetix) and C-arm X-ray guidance to position the ureteral stent in an additional four goats (Figure 2). By following this clinical protocol, we managed to successfully position the stent with a minimal increase in surgery time. When the ureteral stent penetrated the renal cortex (Figure 2(b)), we were able to adjust the position until the stent was correctly placed (Figure 2(c)). This way we avoided unwanted side effects and the regeneration of the ureter progressed as initially intended in the experiment.

It is likely that other groups in different fields have similar experiences with the use of medical devices in their experiments. Based on our experience with ureteral stenting in goats, we advise to always use routinely applied clinical techniques when using medical devices in animals. This refinement is often fast and easy to perform and will prevent unnecessary suffering of animals and reduce the number of animals required due to technical failures.

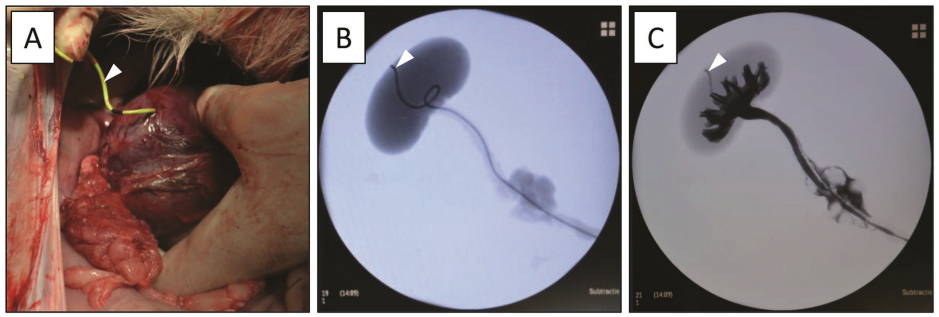

Figure 1. a. Macroscopic view of the right kidney after perforation by the ureteral catheter (white arrowhead). **b**. X-ray scan without iodinated contrast fluids. Stent is clearly visible in the system and perforates the renal cortex (white arrowhead). **c**. X-ray scan with iodinated contrast fluids. Contrast fills the kidney calculi and the stent can be seen in the renal cortex outside of the calculi (white arrowhead).

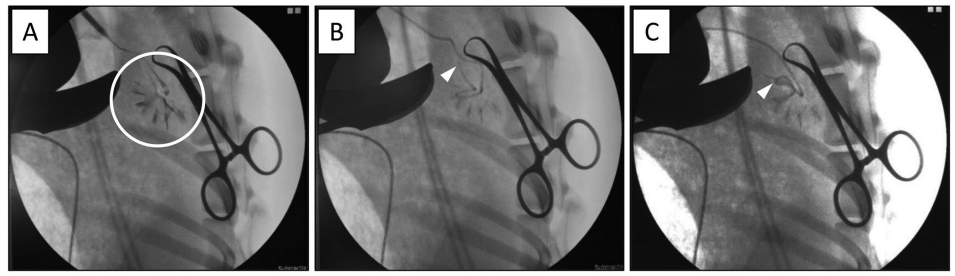

Figure 2.a. X-ray scan of the right kidney (white circle) with iodinated contrast fluids to visualize the renal calculi before inserting the stent during surgery. **b**. After inserting the stent, the tip was perforating the renal cortex (white arrowhead), indicating a misplaced stent. **c**. The stent was retracted and repositioned. This time the stent curled nicely inside the calculi (white arrowhead) and no penetration of the renal cortex was seen, confirming successful stent placement.

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Chapter 9

Ureteral reconstruction in goats using tissue engineered templates and subcutaneous pre-implantation

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Abstract

Introduction: Repair of long ureteral defects often requires long graft tissues and extensive surgery. This is associated with complications including a lack of suitable tissue and graft site morbidity. Tissue engineering may provide an attractive alternative to the autologous graft tissues. In this study, ureteral repair using (pre-implanted) tubular collagen-Vicryl templates was evaluated in a new goat model.

Materials and methods: Tubular templates were prepared from tubularized Vicryl meshes and 0.7% type-I collagen (length = 6 cm, inner diameter = 6 mm, wall thickness = 3 mm). In total, twelve goats were used and evaluated after three months. Eight goats were implanted with the collagen-Vicryl templates and in four goats the templates were first pre-implanted in the subcutis and subsequently used as ureteral graft.

Results: Template implantation was successful in 92% of the goats (11/12). During follow-up, 82% of the animals (9/11) survived without signs of discomfort. Two animals were sacrificed prematurely due to kidney perforation by the stent and urine leakage. Two other animals presented with stenosis of the neo-ureter due to stent migration. After pre-implantation, the templates were remodeled mostly to autologous tissue with similar mechanical characteristics as the native ureter. Goats grafted with pre-implanted templates presented with predominantly healthy kidneys, while the goats grafted with the collagen-Vicryl templates presented with fibrotic and inflamed regions in the kidneys.

Conclusion: The use of pre-implanted tissue templates showed favorable results compared to direct functional implantation of the templates. Partial remodeling towards autologous tissue and similar mechanical characteristics likely improved the integration in the ureteral tissue. Pre-implantation of tissue engineered templates should therefore be considered when two-stage procedures using a nephrostomy catheter are indicated or when planning allows for additional time to treatment.

Introduction

Ureteral trauma can lead to severe problems, including urinoma, sepsis and even kidney loss. While these traumas are relatively rare, they do account for approximately 3% of all urogenital traumas. Iatrogenic injury is the main cause, with hysterectomy during gynecological surgeries accounting for about 73% of all injuries to the ureter [1-3]. Primary repair is often possible when the injury is recognized, but in 33-88% of the cases injury is not recognized during the surgery [4, 5]. Depending on the time between treatment, the type, and the location of the injury, different treatment options are available. Primary end-to-end anastomosis is often only possible shortly after the injury or in very short distance injuries. More severe damage requires extensive and challenging procedures, such as a Boari flap, transureteroureterostomy or ileal interposition [6, 7]. Especially when donor tissue like the ileum is used, the complication rate is as high as 40% and almost all patients need an intervention to treat obstruction after surgery [8], emphasizing the need for alternative approaches. Tissue engineering may be a new source of graft tissue, but in the past 3 decades failure rates have been high, despite the use of many biomaterials and approaches [9].

Collagen is a biomaterial of interest because of its availability, plasticity and low antigenicity [10]. In previous studies in pigs we evaluated tubular templates prepared from collagen only, with or without cells and reinforced collagen-Vicryl tubes, with or without growth factors to repair ureteral deficits. While regeneration of the ureter was possible, the kidneys suffered from the procedure. Rapid wound healing and strong fibrosis were hypothesized to be the main factors causing hydronephrosis [11, 12]. Interestingly, in a recent urostomy study in pigs pre-implanted and subsequently translocated templates showed a more favorable outcome than direct implantation [13]. In the current study, mature goats with a slower wound healing process were used to study (pre-implanted) collagen-Vicryl templates for ureter tissue engineering.

Methods

Template preparation

Vicryl meshes (Ethicon) were tubularized around a stainless-steel mandrel with Vicryl sutures (Ø = 8 mm). Tubular 0.7% (w/v) type I collagen (Collagen solutions, Eden Praire, USA) –Vicryl templates (l = 7 cm, lumen Ø = 6 mm, wall = 3 mm) were prepared as follows: homogenized collagen was cast in a cylindrical mold. Next, the Vicryl mesh and a 6 mm stainless-steel mandrel was inserted, followed by freezing and freeze-drying as described [14]. After lyophilization the collagen was cross-linked using Carbodiimide [15] and final hybrid templates were lyophilized a second time and packaged in blisters. All production steps were performed in a commercial cleanroom (EMCM). The final product was sterilized by ethylene oxide (Synergy Health). The degree of cross-linking was determined using a 2,4,6-trinitrobenzene sulfonic acid assay in triplicate. Template ultra-structure was characterized by scanning electron microscopy (JEOL JSM-6310).

Mechanical characterization

Tensile ring tests were performed to investigate the (bio)mechanical properties of the hybrid templates and autologous tissues. Wet ring specimens of l: 10mm of hybrid templates (N = 4), pre-implanted templates (N = 4) and midsections of goat ureteral tissue (N = 9) were mounted between customized hooks (hook-to-hook distance: 6mm) of a tensile tester (Z2.5 TN, Zwick/Roell) equipped with a 2.5kN load cell. Test specimens were pre-conditioned radially (50mm/min) by stretching 10x to 50% strain. Afterwards, uniaxial load was applied until rupture. Force/displacement data were normalized to the test specimen dimensions to compute a stress/strain curve. The initial elastic modulus was calculated from the first slope of the curve. The ultimate tensile strength (UTS) and maximum strain at break were defined as the maximum stress and strain before failure. Data analysis was performed with SPSS statistics version 22 (IBM) using the one-way ANOVA test and Bonferroni post hoc analysis. $P<0.05$ was considered statistically significant.

Animals

The Nijmegen Medical Center animal ethics committee approved the study protocol (RU-DEC-2014-223). All procedures were performed according to the Institute of Laboratory Animal Research guide for Laboratory Animals [16]. In total twelve mature female Saanen goats, weighing 45-65 kg were used. They were housed in groups at the Institute's farm with a restricted diet and free access to water. The goats were transported to the animal laboratory the day before every procedure. Eight goats received a hybrid template, while in four other goats the template was pre-implanted in the subcutis first. Goats were sedated trough intravenous injection of 5 µg/kg medetomidine (Domitor, Orion Pharma) and 5 µg/kg propofol (Fresenius Kabi). Goats were intubated and anesthesia was maintained using 1,5% isoflurane (Pharmachemie). Subcutaneous injection of 0,5 mg/kg Meloxicam (Novem, Boehringer Ingelheim) was administered for analgesia. Intravenous antibiotics were administered (Amoxicilline, 10 mg/kg, Aurobindo pharma). During recovery, intra muscular injections of 5 µg/kg buprenorphine (AST Farma), 10.000 IE/kg benzylpenicilline (Procpen 30, DoPharma) and 15 µg/kg atipamezole (Orion Pharma) were given. Post-operatively, subcutaneous injection of 0,5 mg/kg Meloxicam and intramuscular injection of 10.000 IE/kg benzylpenicilline was given daily for three days.

Surgical procedure

Pre-implantation

One month before ureter reconstruction, two hybrid templates (l = 7 cm) were subcutaneously implanted in four goats on the right flank. A 5-cm incision was made approximately 3 cm from the lower rib. Two separate pockets were created by spreading the subcutaneous tissue using long scissors towards the hind leg. Hybrid templates were placed over a silicon tube (Ø = 6 mm) and were fixed to the tube using a 2-0 Vicryl suture on both ends. Next, the hybrid templates were placed and fixed in the pockets using 2-0 Vicryl sutures. Finally, the subcutis and skin were closed using 2-0 Vicryl sutures.

Functional implantation

A flank incision (l = 10 cm) was made in the right flank of the goats 5 cm below the spine in parallel to the lower rib next to the pre-implantation scar. The muscle layers were separated in the direction of the muscle fibers to enter the retroperitoneal space. The ureter was located and mobilized. Next, the ureter was stripped from the fatty layers for better visibility. The ureter was transected 5-11 cm from the kidney and both ends were spatulated. Hybrid templates or pre-implanted templates (l = 6 cm) were placed over a 4.7 Fr/22-32 cm multi-length double-J stent (Inlay Optima, Bard medical) followed by stent positioning in the kidney and bladder. Following technical complications related to stent placement, a C-arm was used to determine correct positioning of the stent in the kidneys for the final five goats [17]. A tension free anastomosis was created using 6-0 monocryl sutures (Ethicon). A 6-0 prolene suture placed in the ureter approximately 1-cm from the anastomosis site on both sides as markers. Fibrin glue (Tisseel, Baxter) was used to cover the anastomosis sites and templates to prevent leakage. The individual muscle layers, the subcutis and skin were closed (2-0 Vicryl). The animals were followed closely for signs of discomfort.

Blood tests

After observed technical complications, blood creatinine, CRP and sedimentation was analyzed in four animals to detect possible kidney failure and inflammation. Samples were retrieved pre-operatively, post-operatively and then weekly for up to one month. Samples were analyzed by the clinical chemistry lab of the Radboud university medical center.

Evaluation

The goats were sacrificed by an overdose of intravenous pentobarbital approximately three months after the implantation procedure to study medium-term tissue regeneration. The urinary tract was harvested and evaluated macroscopically. Next, the size of the internal ureter and kidney morphology was investigated by radiology using iodinated contrast fluid (Iomeron 300, Bracco Imaging). Finally, tissue specimens of the neo-ureter, ureters, kidneys and urinary bladder were harvested for histological evaluation. Specimens were fixed in 4% (v/v) formaldehyde in PBS (Boom) overnight and embedded in paraffin. The goats in which kidney perforation occurred with extensive urine leakage had unreliable tissue regeneration results and were omitted from further analysis (Table 1).

Immunohistochemistry

Sections of 4 µm were cut and stained with hematoxylin (Klinipath) and eosin (Merck) for morphological analysis. Pre-implanted templates and neo-ureter tissue after grafting were stained for pancytokeratin (AE1/AE3, Fisher Scientific), desmin (33, Biogenix), α-smooth muscle actin (αSMA, 1A4, Biogenix) and Verhoeff/

Masson's Trichrome (Pathology, Radboudumc). In brief, antigen retrieval was performed using microwave treatment in sodium citrate (pH 6, 10 min boiling and subsequently cooling to RT) for pancytokeratin immunohistochemistry (IHC). Peroxidase activity was blocked by incubation in 1% (v/v) H_2O_2 (Merck) in PBS for 30 min. Next, sections were incubated with 10% (v/v) rabbit serum for 10 min, followed by 1 h incubation with primary antibodies: pancytokeratin (1:400), desmin (1:200), αSMA (1:16000). Secondary antibody (polyclonal rabbit anti-mouse immunoglobulins/HRP (Dako) was incubated for 30 min (1:100). Then, 3,3'-Diaminobenzidine (bright-DAB, Immunologic) was used to develop the slides. Finally, sections were counterstained using hematoxylin for 5s. All antibodies and goat serum were diluted in PBS containing 1% (v/v) bovine serum albumin (BSA, Sigma-Aldrich).

Qualitative analysis

Pre-implanted tissues were investigated for template degradation, inflammation and vascularization. Kidney sections were investigated for the occurrence of inflammation and fibrosis, morphology of tubuli and glomeruli. Neo-ureter sections were investigated for template degradation, tissue development (muscle, urothelium, vasculature), fibrosis and inflammation. All sections were evaluated in a blinded fashion.

Results

Templates

Structure

SEM analysis showed highly porous honeycomb structures typical for collagen-based templates (Figure 1). The tubularized Vicryl meshes were successfully integrated in the collagen as evidenced by collagen attaching to the Vicryl. Carbodiimide cross-linking stabilized the collagen resulting in a 49% free amine group reduction. After pre-implantation, the templates were remodeled by host tissue and well vascularized. Collagen and Vicryl were partially degraded and a dense tissue layer formed on the inside and outside surrounding the remnants of the templates (Figure 1).

Mechanical properties

All templates showed a "J" shaped stress-strain curve in tensile ring tests (supplementary figure 1). Templates showed an initial high elasticity followed by an uprising slope indicative of a rising stiffness before total failure. Hybrid templates showed a significantly higher initial ($p<0.0001$) and ultimate tensile strength ($p<0.0001$) compared to the other templates (figure 2). The difference between the initial tensile strength and ultimate tensile strength of pre-implanted templates and goat ureteral segments was not significant ($p=0.8$ and $p=0.13$ respectively).

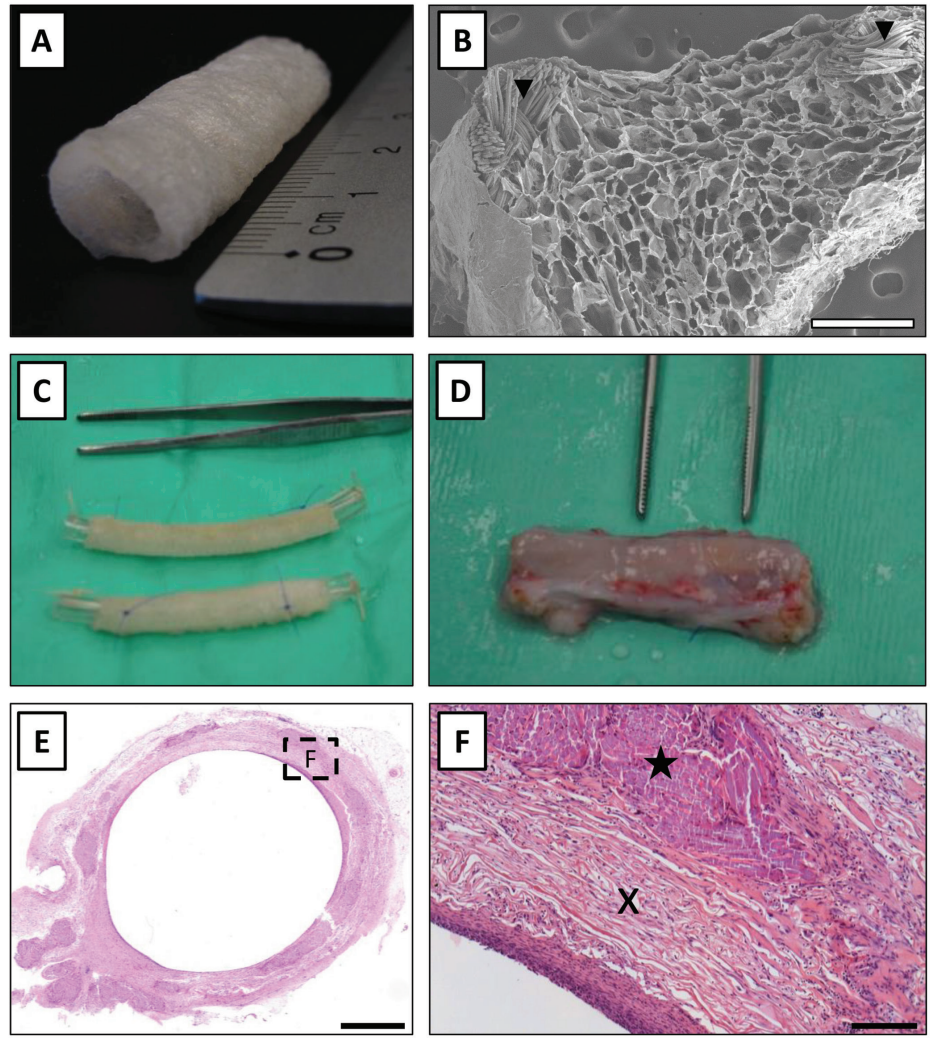

Figure 1. Material characterization. A. Macroscopic view of a dry collagen-Vicryl template. B. Cross-sectional scanning electron microscopy image of a collagen-Vicryl template showing an open porous structure and good integration of the Vicryl mesh (arrow head). Scalebar = 500μm. C. Templates were placed over a silicon mandrel before pre-implantation in the subcutis. D. Macroscopic view after one month of pre-implantation in the subcutis showing tissue encapsulation and vascularization. E. Histological cross-section of pre-implanted template. Scalebar = 2000μm. F. Magnification of cross-sectional overview. * indicates the Vicryl mesh. X indicates collagen remnants. A dense tissue layer formed on the inside, sealing the template. Scalebar = 250μm.

Animal surgery

Success rate of the implantation procedure was 92% (11/12). One animal was sacrificed during surgery as the ureter wall ruptured and the ureter retracted, resulting in a defect >10 cm that could not be repaired. During follow-up, 82% (9/11) survived without signs of discomfort. Two animals were sacrificed before the predetermined endpoint of three months due to rapid weight loss and signs

of discomfort. Upon necropsy kidney perforation by the stent and subsequent urine leakage from the kidney was observed. In two animals that reached the endpoint, the stent had perforated the kidney. One animal belonged to the group with hybrid templates, while the other received a pre-implanted template. Based on this experience, x-ray guided stent placement was introduced in the next five animals, after which no kidney perforation was observed. Two of these animals presented with stent migration which led to stenosis of the neo-ureter. Five animals had no major complications. Results per animal are summarized in table 1. Figure 3 shows the templates during implantation surgery.

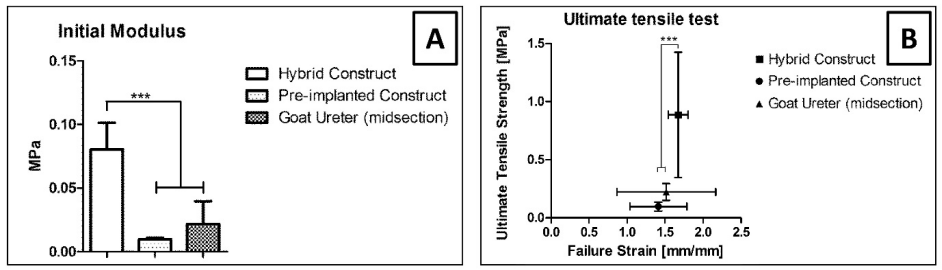

Figure 2. Biomechanical analysis. **A**. Initial modulus of collagen-Vicryl hybrid template, pre-implanted template and native tissue. Hybrid templates show a significantly higher initial modulus compared to pre-implanted ($p<0.0001$) and native tissue ($p<0.0001$). No difference was found between pre-implanted templates and native tissue ($p=0.8$). **B**. Ultimate tensile strength and strain at the failure point of collagen-Vicryl hybrid template, pre-implanted template and native tissue. Hybrid templates show a significantly higher ultimate tensile strength compared to pre-implanted ($p<0.0001$) and native tissue ($p<0.0001$). No difference was found between pre-implanted templates and native tissue ($p=0.13$). No differences were found regarding strain at failure for any of the specimens ($p>0.99$).

Macroscopic evaluation

The appearance of the right kidneys was normal, regardless of the treatment modality (Figure 4A-B, F). The shape, size and color resembled the control kidneys, albeit that small patches of dyschromia were observed sporadically, as well as a slightly thicker renal capsule. The proximal ureter was slightly dilated and the area of implantation showed signs of fibrosis in the animals grafted with the hybrid templates. The size of the regenerated tissue was approximately 1.5 cm in both groups compared to the 6 cm graft. Small diverticula were observed at the anastomosis site when hybrid templates were used. Additionally, when hybrid templates were used collagen remnants were found between the ureteral stent and the new tissue which resembled a gel like substance. When pre-implanted templates were used remnants were absent.

X-ray evaluation

No differences between the appearance of the right kidney connected to the reconstructed ureter and the left (control) kidney was observed (Figure 4). The calyces were clearly distinguishable. The pyelum and proximal ureter were slightly dilated on the treated side. Small diverticula were observed in the group without pre-implantation at the anastomosis site.

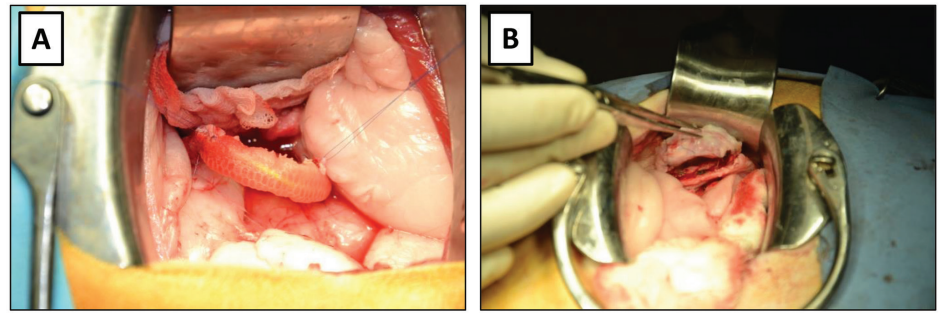

Figure 3. Surgical procedure. **A**. Implantation of a collagen-Vicryl template between two spatulated ureter ends. **B**. Implantation of template between two spatulated ureter ends after pre-implantation.

Blood analysis

Blood creatinine was slightly elevated (95.0 ± 8.5 µmol/l) in all analyzed animals as a result of the surgery. Creatinine levels returned to normal (64.8 ± 10.1 µmol/l, based on pre-operative measurements) in all goats in the week thereafter. No changes were found for CRP (<1 mg/l) and sedimentation rate (2 mm/hour) at any time point in any of the analyzed goats.

Histological evaluation

Three goats could be included for histology in both groups (table 2). The other goats had compromised regeneration due to complications with stenting (kidney perforation or stent migration), which resulted in excessive urine leakage (kidney perforation) or stenosis of the ureter (stent migration) and were therefore excluded from further analysis.

Hybrid template group

Kidneys

Morphological analysis of the kidney connected to the reconstructed ureter revealed fibrotic areas and inflammation in the renal cortex (Figure 4G). In two goats, fibrosis and inflammation was also observed near the calyces.

Neo-ureter

Integration of the hybrid template was poor (Figure 5A). Although only a small amount of template remnants was found in the neo-ureter, many remnants were found extruded in the ureteral lumen (Figure 5B). The newly formed tissue consisted mainly of connective tissue with α-sma positive cells in parallel alignment to the ureteral lumen and extensive vascularization. Epithelial lining was absent as judged by the lack of panCK-positive cells (Figure 5C,D). Smooth muscle cell ingrowth was limited to the anastomosis sites as evidenced by the minimal amount of desmin positive cells growing into the neo-ureter (Figure 5E,F). A small diverticulum (<1 cm^2) was observed in two animals near the anastomosis site.

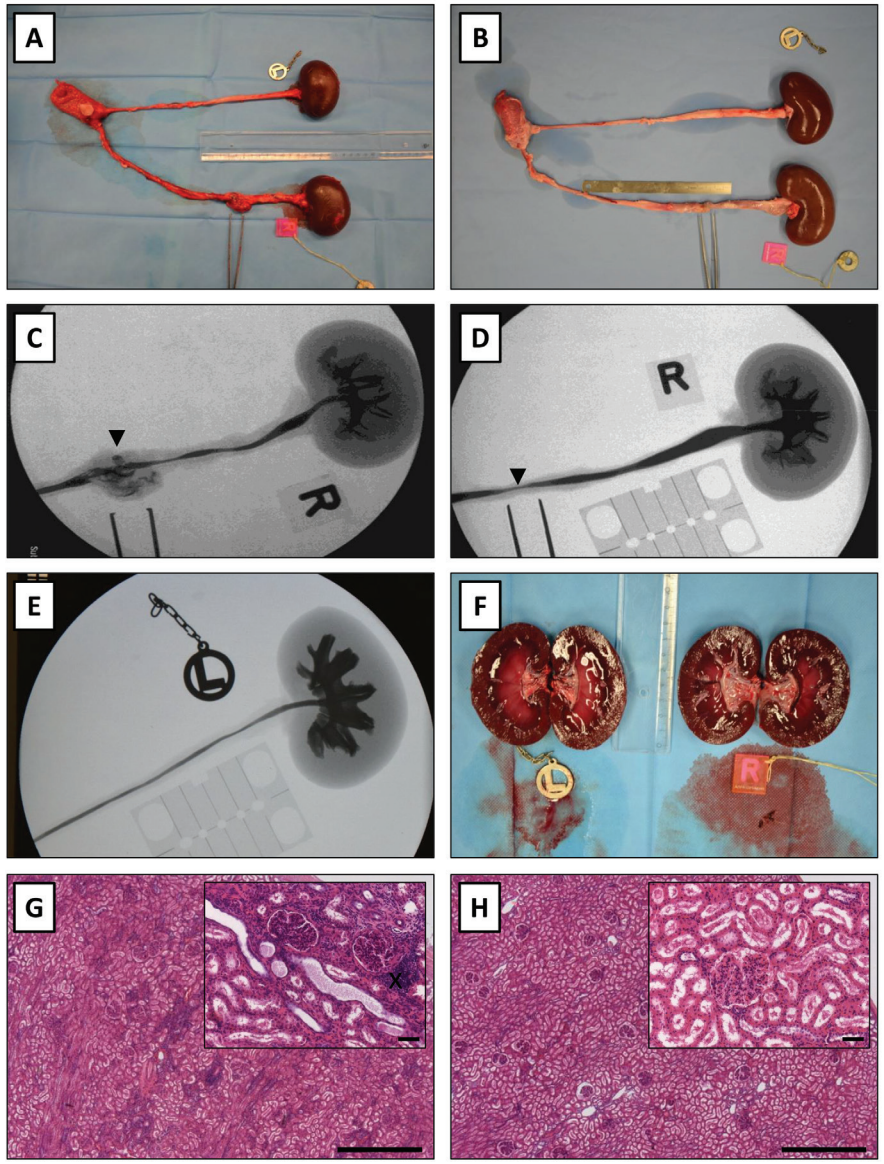

Figure 4. Evaluation overview 3 months after implantation. **A**. Macroscopic view of the urological tract after implantation of a collagen-Vicryl template in the right ureter. **B**. Macroscopic view of the urological tract after implantation of a pre-implanted template in the right ureter. **C**. X-ray image of the ureter and kidney after implantation of a collagen-Vicryl template. Arrowhead shows implantation site. **D**. X-ray image of the ureter and kidney after implantation of a pre-implanted template. Arrowhead shows implantation site. **E**. X-ray image of left ureter and kidney (control). **F**. Cross-section of both kidneys after implantation of a pre-implanted template showing no differences between treated and control kidney. **G.** Histological view of the kidney after implantation of a collagen-Vicryl template showing influx of inflammatory cells (X). **H**. Histological view of the kidney after implantation of a pre-implanted template showing healthy kidney tissue. G,H. scalebar = 250 µm.

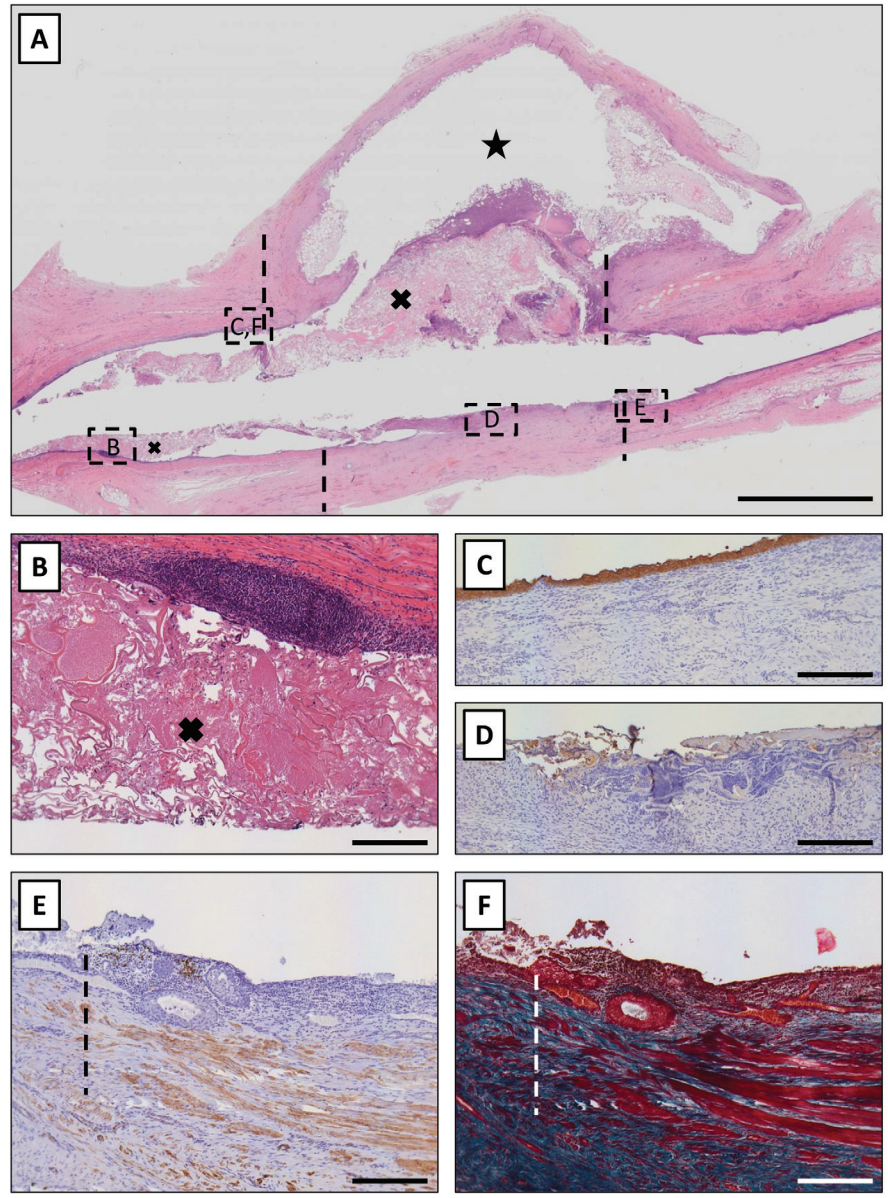

Figure 5. Histological analysis 3 months after implantation of a collagen-Vicryl template. **A**. Overview of the regenerated area. Newly formed tissue can be found between dashed lines. * indicates a small diverticulum. X indicates template remnants. Scalebar = 5 mm. **B**. Collagen remnants were found in the lumen of the ureter as indicated by X. **C**. PanCK staining of normal tissue. **D**. PanCK staining in the regenerated tissue. PanCK-positive cells were sparsely present. **E**. Desmin staining at the anastomosis site (native tissue right of dashed line, new tissue left of dashed line) indicates limited muscle cell ingrowth. **F**. Masson-Verhoeff staining confirms limited muscle ingrowth as muscle morphology changes from spindle like bundles in the native tissue (right) to small patches in the new tissue (left). Muscle and epithelium in red, ECM in green. B-F. Scalebar = 250 µm.

Pre-implantation group

Kidneys

Morphologically the kidney tissue on the treated side was similar to native kidney tissue of the untreated side (Figure 4H). The tubuli and glomeruli were almost completely intact and changes were minimal. In one goat slight influx of inflammatory cells was observed in the renal cortex. In another goat slight inflammation in one of the calyces was seen, while in the third goat inflammation was absent.

Neo-ureter

The pre-implanted template integrated well in the ureter. The newly formed ureter tissue had a similar wall thickness compared to the native ureter (Figure 6A). Smooth muscle tissue ingrowth was limited to the anastomosis sites as evidenced by minimal desmin positive cell ingrowth into the neo-ureter (Figure 6C). A single layered epithelium was observed throughout most of the luminal area as indicated by panCK-positive cells (Figure 6B,D). The new tissue consisted mainly of connective tissue with α-sma positive cells and extensive vascularization (Figure 6E). There was mild inflammation, particularly around the anastomosis site. The template was mostly degraded as evidenced by the minimal collagen and Vicryl remnants (table 2). No diverticulum was observed in any of the animals.

Discussion

There is a clear need for alternative treatments options for large ureteral defects when conventional surgical techniques cannot be used or donor tissues is not available. Nevertheless, ureteral tissue engineering is an understudied field in urogenital tissue engineering, possibly due to the lower number of patients in need for alternatives compared to other urogenital tissues such as the urethra or the urinary bladder [18, 19]. In this study we repaired an induced ureteral defect with (pre-implanted) hybrid templates in adult goats. The main goal of a ureter reconstruction is to salvage the disconnected kidney to prevent kidney loss and restore the urinary tract. Therefore, the most important outcome of successful ureteral reconstruction is minimal kidney damage. In this large animal model we show that ureteral defect can be repaired with a tissue engineering approach (6 cm long implant) with presumably minimal damage to the kidney. After ureteral repair with either direct grafting of a hybrid template or after pre-implantation of this template, blood creatinine levels returned to pre-operative values within one week and remained stable afterwards, indicating good overall kidney function. However, morphological analysis of the tissue with a pre-implanted template were superior to direct implantation of hybrid templates, suggesting that pre-implantation followed by translocation is superior. Although blood creatinine levels normalized and histological analysis showed minimal kidney damage after reconstruction, future studies should include comprehensive functional studies, e.g. measure the glomerular filtration rate (GFR) to show potential differences in clearing rate between the left and right kidney [20]. Moreover, although follow-up

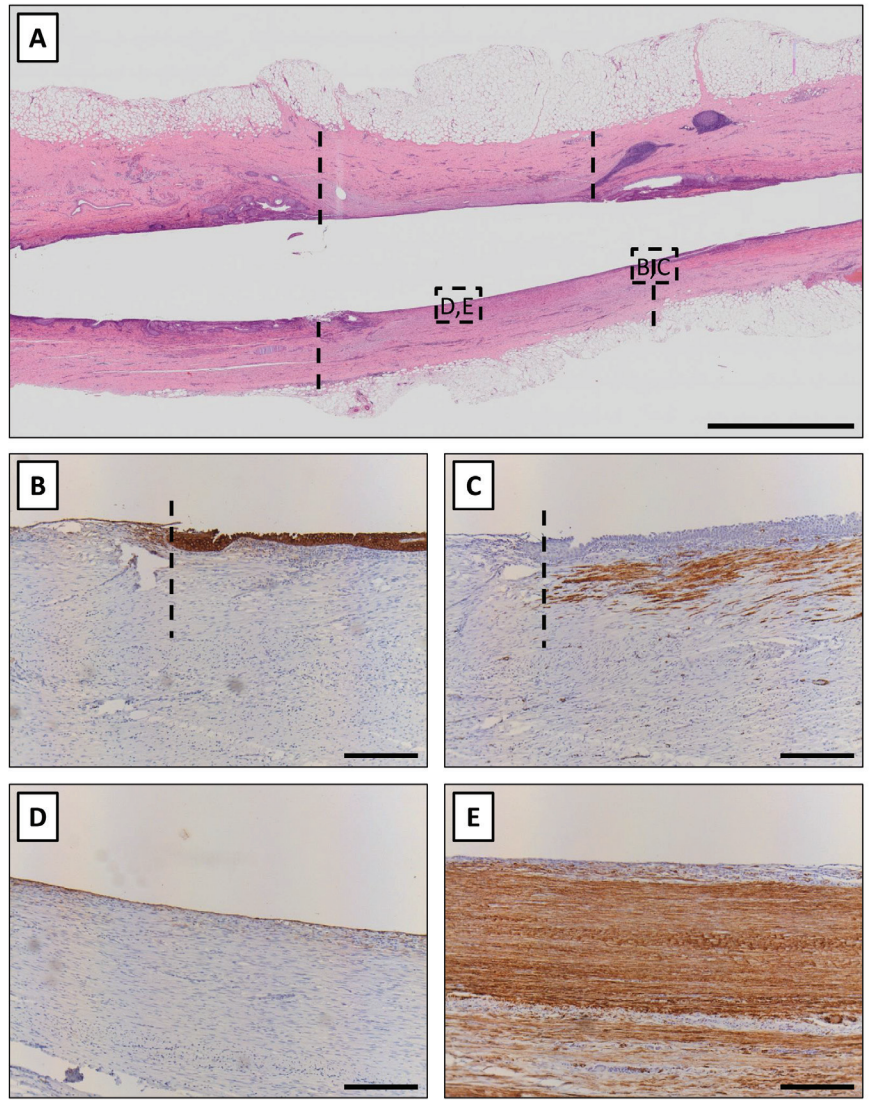

Figure 6. Histological analysis 3 months after implantation of a pre-implanted template. **A**. Overview of the regenerated area. Newly formed tissue can be found between dashed lines. Scalebar = 5 mm. **B**. PanCK staining at the anastomosis site. Morphology changed from normal to single layered epithelium. **C**. Desmin staining at the anastomosis site indicates limited muscle cell ingrowth. **D**. PanCK staining in the regenerated tissue. A single layered epithelium was observed throughout most of the regenerated tissue. **E**. αSMA staining in the regenerated tissue shows alignment of fibroblasts in the extracellular matrix. B-E. Scalebar = 250 µm.

of animals was twelve weeks, a reasonably long follow-up period, true long-term follow-up of at least one year is needed to adequately mimic the human situation.

Pre-implantation procedures have previously shown promising results in tissue engineering, including ureteral reconstruction [21, 22]. Here we show the ability to create tissue tubes that were well vascularized and in which the initial

inflammatory response had largely subsided before functional implantation. Through the insertion of a silicon mandrel the lumen was maintained during this initial remodeling, preventing a change in template dimensions. After functional implantation, the connective tissue that covered the lumen of the neo-tissue templates may have protected the surrounding tissue from the toxic urine, preventing absorption of urine into the template remnants [23]. Without pre-implantation, the template can act as a sponge that absorbs urine which may have a detrimental effect on the remodeling process by constantly irritating the surrounding tissue. After the remodeling phase the pre-implanted tissue had similar mechanical properties compared to the native ureter in contrast to the significantly stiffer hybrid templates. This higher stiffness may have led to a compliance mismatch that can cause local tissue stiffening and disturbed flow profiles resulting in hyperplasia [24, 25]. The combination of a high stiffness and constant exposure of the regenerating tissue to urine may have contributed to the formation of diverticula, extrusion of collagen into the lumen and slower remodeling of the tissue. The compromised regeneration may also influence kidney outcome due to higher ureteral pressures.

Interestingly, collagen sponges have previously shown good results in other urogenital tissue engineering applications, such as urethra replacement and urinary bladder augmentation [26, 27]. This difference may be explained by constant exposure to urine in the ureter compared to intermittent exposure during voiding in the urethra and folding of the urinary bladder when empty. Moreover, the anatomical characteristics are different, e.g. the urethra is tightly surrounded by fascia and other penile tissues while the ureter is positioned freely in the retroperitoneal space which makes it susceptible to mechanical stresses.

Here we studied adult goats instead of young pigs and showed a much more favorable outcome regarding the kidney. Due to their size, we previously used young pigs (3-6 months old) for ureter reconstruction studies [28]. Their fast growth and rapid wound healing may have negatively impacted the remodeling of the neo-ureter and resulted in hydronephrosis; an undesired outcome. Adult goats have a similar size and weight to these young pigs, but have the wound healing capacity of adult animals. By using adult animals, we may have better mimicked adult human tissue regeneration.

Smooth muscle tissue regeneration in the neo-ureter was limited compared to pigs. While smooth muscle tissue is important for ureter functionality, the regeneration of this tissue after ureteral defect repair may not be as important as initially hypothesized when only part of the contractile function is lost. In addition, the presence of the double-J catheter may have made muscle regeneration redundant as it guarantees urine flow. Therefore, this catheter should be removed when studying longer time points. In the current study, the pre-implanted tissue tube functioned adequately as ureter segment to maintain kidney integrity and function within the study time frame. Interestingly, graft shrinkage from 6 to 1.5 cm length had no detrimental effect on the kidneys. This may be related to the greater mobility of goats compared to pigs which may improve the flow of urine. In general, the goat model appears to be a good alternative to the pig model when studying ureter reconstruction. However, the size of the kidney pyelum and the inner diameter of the ureter are much smaller than that of humans and pigs. In contrast to previous studies, no resistance was noticed when the guidewire perforated the kidney. Consequently, double-J stent positioning was problematic in some cases, necessitating the use of human clinical protocol levels using X-ray and iodinated contrast fluids [17]. Additionally, the small size of the ureter complicated anastomosis of the relatively oversized templates to the ureter. Smaller diameter templates may need to be considered for future experiments to better match template dimensions to the ureter.

While pre-implantation of the templates showed promising results for ureteral repair, this method has a major drawback: In general, this type of reconstruction is mostly unforeseen and requires fast intervention. I.e., it may not be possible to pre-implant a template and wait for one month in all patients. Nevertheless, for severe and long ureteral defects a two-stage repair using a percutaneous nephrostomy catheter to drain urine during is a standard treatment option [29]. The time between the two stages could be used for pre-implantation. However, investigating this method is complicated in animals due to their mobility which may lead to early elimination of the nephrostomy catheter. Alternatively, different templates that do not require pre-implantation to be watertight and mimic the native tissue properties could provide a solution. Decellularization of native tissues may be an attractive alternative to investigate [30-32]. In addition to being watertight and having similar tissue properties, beneficial bio-active compounds such as growth factors may remain in the tissue to stimulate wound healing and remodeling.

Conclusion

Ureteral reconstruction using pre-implanted hybrid templates is superior to direct grafting in a goat model. This study showed that matching mechanical characteristics to the native tissue is superior to using stiffer templates and leads to better wound healing and tissue regeneration. Pre-implantation of tissue engineered templates should be considered when two-stage procedures are indicated or when the surgery can be planned. Finally, functional kidney analysis should be performed and longer follow-up should be studied to determine long-term outcome of ureteral defect repair using tissue engineering.

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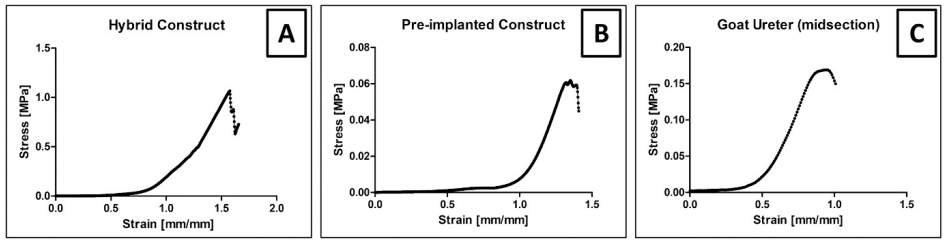
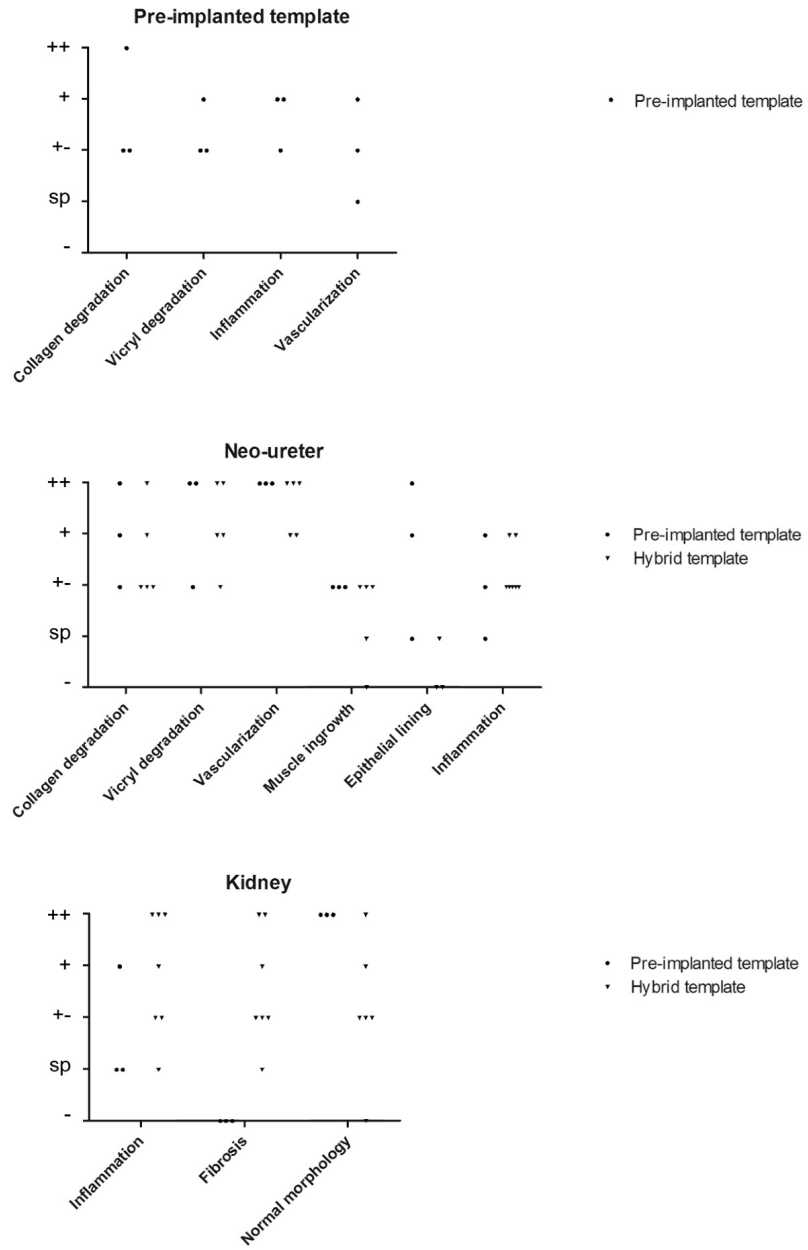


Supplementary figure 1. Representative stress-strain curves of the tensile ring tests showing a typical "J" shape. **A**. Hybrid construct. **B**. Pre-implanted construct. **C.** Goat ureter (midsection).



Supplementary figure 2. Outcome of blinded histological scoring of representative sections of the templates after pre-implantation, after functional implantation and the kidney on the treated side. - = not present, sp =- sporadically present, +- = somewhat present, + = present, ++ = abundant.

	Time in experiment	Survival	Distance implant to kidney	Neo ureter size	Observations	Included for histology
No pre-implantation						
Goat 1	0 days	Sacrificed in surgery	-	-	Ureter ruptured in surgery during suturing, defect become too long to repair	No
Goat 2	84 days	Survived	5 cm	1.5 cm	Double-J penetrated kidney, hydronephrosis	No
Goat 3	67 days	Sacrificed 2 weeks early	8 cm	1.5 cm	Double-J penetrated kidney	No
Goat 4	42 days	Sacrificed 6 weeks early	11 cm	-	Reached humane endpoint (6 kg weight loss, signs of discomfort), Double-J penetrated kidney, urinoma on top of kidney	No
Goat 5#	91 days	Survived	7 cm	1.5 cm	Small diverticulum at anastomosis site (<1cm²)	Yes
Goat 6#	91 days	Survived	7.5 cm	2.0 cm	Small diverticulum at anastomosis site (<1cm²)	Yes
Goat 7#	94 days	Survived	7 cm	1.5 cm	Double-J stent migrated to neo-ureter site, fistula around neo-ureter (5-10cm²), neo-ureter obstructed	No
Goat 8#	94 days	Survived	6 cm	2.0 cm	Double-J stent migrated to bladder, complete obstruction of neo-ureter, weight loss* (20 kg).	Yes
Pre-implantation						
Goat 9	27 + 85 days	Survived	7 cm	3.5 cm	Double-J stent penetrated kidney, urinoma on top of kidney, distal part of the stent in ureteral sheets instead of ureteral lumen	No
Goat 10	28 + 84 days	Survived	7 cm	1.5 cm	No complications	Yes
Goat 11	31 + 81 days	Survived	10.5 cm	1.5 cm	No complications	Yes
Goat 12#	31 + 84 days	Survived	9 cm	2.0 cm	No complications	Yes

Table 1. Outcome of the surgical procedure for each goat. For pre-implantation, the time in experiment is split to show pre-implantation + implantation times in experiment. Technical problems with double-J stents caused them to penetrate the kidney cortex in goats 2,3,4 and 9. These goats were excluded for further analysis as this may have influenced the regeneration results. *Goat 8 unexpectedly appeared to be pregnant during the experiment. Weight loss probably resulted from carrying the young, as no other signs of discomfort were observed. # indicates goats in which x-ray was used for stent positioning.

		No pre-implantation	Pre-implantation
Pre-implanted tissue	Collagen remnants	NA	+
	Vicryl remnants	NA	+-
	Inflammation	NA	+
	Vascularization	NA	+-
New ureter tissue	Collagen remnants	+-	+-
	Vicryl remnants	+-	+-
	Inflammation	+-	+-
	Vascularization	++	++
	Muscle ingrowth	+-	+-
	Epithelial lining	-	+
Kidney	Normal morphology	+-	++
	Inflammation	+	+-
	Fibrosis	+	-

Table 2. Outcome of blinded histological scoring of representative sections of the templates after pre-implantation, after functional implantation and the kidney on the treated side. NA = not applicable, - = not present, +- = somewhat present, + = present, ++ = abundant. Supplementary figure 2 shows a dot plot of the individual scores.

Chapter 10

Summary and future perspectives

Summary

Reconstruction of large urogenital defects remains a challenging procedure that often require donor tissues such as local skin flaps, buccal mucosa or bowel tissue. While repair is often possible, it is associated with severe side effects like donor side morbidity, metabolic abnormalities and recurrences of the original problem. This thesis focused on the development of tissue engineered medical devices as an alternative to autologous donor tissue. Reconstructive surgery using biomaterials was initiated around the seventies. In the nineties, this field started to grow rapidly and the term "tissue engineering" was introduced [1]. Tissue engineering is a fast growing discipline that aims to replace or repair damaged organs and tissue [2]. The engineered constructs temporarily support the damaged organ or tissue and ideally are gradually replaced during wound healing and tissue remodeling. Depending on the tissue, the constructs can be designed to specifically meet the requirements of the local environment such as mechanical properties and the degradation speed. Although many approaches, including different biomaterials, cells and growth factors have been studied since then, clinical translation for urogenital applications is still limited. Only in rare cases, where routine clinical practice has failed repeatedly, are tissue engineered alternatives considered [3-5]. This thesis described the current state-of-art for ureteral and urethral tissue engineering, followed by the development and evaluation of new flat and tubular collagen based constructs.

A (systematic) literature overview of the state-of-art

The first part of the thesis provides an overview of ureteral tissue engineering in animal models, followed by a complete overview of urethral tissue engineering in animal models and patients.

Chapter 2 shows that initial attempts at reconstructing the ureter involved large animal models using pigs and dogs. Large defects were created followed by repair using collagen sponges, decellularized bowel tissue and even non-degradable Gore-Tex. The constructs were mostly implanted without seeding cells. While there was evidence of tissue regeneration as evidenced by urothelial and muscle cell regrowth, most animals presented with fibrosis of the ureter causing strictures and hydronephrosis. These results show that ureteral reconstruction may be viable using tissue engineering, but new methods are required to overcome current complications.

In **chapter 3** recent advances in ureteral tissue engineering are reviewed, showing a shift from acellular tissue towards the application of cells on decellularized tissue and collagen sponges. The use of mesenchymal stem cells showed promising results compared to primary cells. Additionally, pre-implantation at a non-functional location improved vascularization and stem cell differentiation. The low number of new publications shows that ureteral reconstruction remains challenging and understudied.

Interestingly, clinical application of urogenital tissue engineering is rare even though there is extensive pre-clinical literature for organs like the urethra. In **chapter 4**, a systematic review on urethral tissue engineering was performed to investigate why clinical translation is limited and which approaches proved to be most successful. This review provided a comprehensive overview of all published literature followed by meta-analysis to study the effect of different approaches. Remarkably, the use of cells significantly reduced the chance of developing side effects in animal models, but not in clinical studies. Other design choices, such as the type of biomaterial or the animal model did not significantly affect the chance for developing side effects, having a functional urethra or completing the study. Quality analysis of the studies revealed that pre-clinical study design needs to improve to better facilitate clinical translation. Proper control groups, as well as blinding and randomization need to be applied. In addition, the outcome of each individual animal should be reported.

New hybrid biomaterials in urogenital tissue engineering

In the second part, the use of newly developed tubular and flat collagen based (hybrid) constructs *in vivo* and *in vitro* is presented. In **chapter 5** the effect of mechanical stimulation on the proliferation and differentiation of primary porcine smooth muscle and urothelial cells was studied. Primary cells were isolated from the urinary bladder of pigs and cultured on flat collagen scaffolds. Static culture was compared to dynamic culture in a bioreactor system. This system simulated the filling and voiding of the bladder to provide a mechanical strain to the cells. Dynamic culture resulted in higher proliferation and alignment of smooth muscle cells. No significant differences between smooth muscle cell specific marker expression was observed and no differences for any outcome was observed for urothelial cells. Considering the alignment, preconditioning of templates with smooth muscle cells under dynamic conditions may improve smooth muscle cell ingrowth in tissue engineered tissues, which is currently a major challenge.

Besides the ingrowth of smooth muscle cells, vascularization of the constructs is a major challenge as there is limited time before necrosis occurs. In **chapter 6** multiple smaller flat collagen scaffolds were implanted into the bladder of pigs instead of one large scaffold to increase the total bladder volume. The use of multiple smaller scaffolds instead of one large scaffold reduced the distance of tissue ingrowth while facilitating a similar increase in bladder surface. To further enhance the vascularization of these scaffolds, growth factors were included using heparin. Primary closure, collagen only and collagen with heparin scaffolds were used as controls to study the effect of adding growth factors. Implantation of multiple flat collagen scaffolds showed to be a feasible technique for bladder augmentation. The largest surface area of regenerated tissue was accomplished using collagen and heparin scaffolds without growth factors. No differences were observed after adding growth factors, which was likely due to a burst release and fast growth factor wash-out due to continuous urine exposure.

Tubular collagen constructs were developed for ureteral reconstruction. An initial trial in pigs (**chapter 4**) revealed the need to enhance the mechanical properties of the collagen. To improve the tensile strength of collagen, knitted Vicryl meshes were incorporated (**chapter 7**). In addition, growth factors were added to improve the vascularization. The Vicryl meshes greatly improved the

mechanical properties of the scaffolds avoiding complications related to scaffold rupture. The increased load of synthetic material did result in a more pronounced immune response, causing excessive fibrosis and hydronephrosis. Similar to the use of growth factors in the bladder augmentation study, no significant long term beneficial effect was observed when growth factors were added. This suggests the need for optimization of growth factor delivery and local suppression of the immune system.

While ureteral reconstruction was possible in the pig model, there were complications that may have been specifically related to the animal choice. For practical reasons, young pigs were used. These pigs grow very fast, which may have influenced the wound healing process and may have led to extensive fibrosis and stricture formation. In **chapter 8** the first experience in adult goats are described. Adult goats were chosen as the new species as they have a similar size and abdominal anatomy to young pigs and humans. Moreover, goats are more mobile than pigs and the use of adult animals better simulates the wound healing of the clinical situation. The surgical procedure was uneventful in the new model, but perforation of the renal cortex by the ureteral stent was observed in four out of seven goats during evaluation after three months. Upon close inspection, the pyelum of the kidneys appeared to be too small for blind stent placement. This necessitated the use of clinical protocols for stent placement, which involves the use of iodinated contrast fluids and X-ray visualization. An additional four goats were successfully treated using these new protocols. This relatively small and easy refinement to the surgical protocol greatly improved the quality of the procedure. It is important to always use clinical protocols when dealing with laboratory animals to avoid unnecessary suffering and reduce the number of animals needed.

Finally, **chapter 9** describes the results of the ureteral reconstruction in goats. Hybrid collagen-Vicryl constructs were implanted in the goat ureter similarly to the pig study (**chapter 7**). Instead of using growth factors, pre-implantation in the subcutis was performed for four weeks to improve vascularization and to enable partial remodeling in a non-functional location. Mechanical properties of pre-implanted tissue were very similar to the native tissue. In contrast, the collagen-Vicryl constructs were much stiffer. Pre-implanted constructs integrated better with the native tissue as, unlike direct implantation, no diverticula or template extrusion into the lumen was observed. Microscopic results were similar regarding smooth muscle regeneration, but urothelium regenerated better after pre-implantation. Moreover, when pre-implanted grafts were used the kidney outcome was superior: Macroscopic and microscopic morphology was predominantly normal, fibrosis was absent and only slight inflammation was observed, compared to a compromised morphology, fibrosis and inflammation when tubular grafts were immediately placed. This may be explained by the better match in tissue properties and the formation of a tissue barrier that may have protected the surrounding tissue from urine. This study showed the importance of matching construct properties to tissue properties and showed the viability of pre-implantation.

Conclusions

This thesis describes the current state-of-art in urogenital tissue engineering by using narrative and systematic reviews. Trends in tissue engineering were identified and suggestions were provided to improve the clinical translation. Importantly, pre-clinical study design needs to be improved, specifically by using proper control groups, blinding and randomization. Furthermore, this thesis describes the development and *in vitro* and *in vivo* evaluation of collagen based tissue constructs for bladder augmentation and ureteral repair. Regenerative outcome was manipulated by either using cells, growth factors or pre-implantation. From these, pre-implantation showed the most promising results and may be required for the successful application of tissue engineering in the clinical setting.

Future perspectives

Translating pre-clinical knowledge to clinical applications is a critical next step in the field of tissue engineering. To reach clinical translation, it is clear that pre-clinical study design should be improved as shown in chapter 4 of this thesis on urethral tissue engineering. Most importantly, future studies should include current standard treatment, such as free skin flaps, buccal mucosa or bowel tissue. Another remarkable observation was the type of tissue engineered constructs that was studied in a clinical setting. Where there is a lot of variety in pre-clinical studies, mainly decellularized tissues without added cells were studied in the clinic. Another important aspect in study design is the duration of the pre-clinical experiments. Currently, most studies have a pre-determined endpoint of 3 or 6 months, which only shows short-term complications. Patients that receive these devices however, need functional organs for many years. Therefore, longer endpoints (> 12 months) should be considered to improve clinical translation. In addition, this allows better understanding of the tissue remodeling over time.

Currently, a lot of effort is put into optimizing biomaterials like collagen to mimic the native tissue properties, e.g. by using different concentrations, by incorporating synthetic materials or by adding growth factors. While tissue engineering allows for diverse construct design, it is next to impossible to mimic native tissue due to its sheer complexity. Decellularized materials may ease this process as the native tissue, including its morphology and mechanical properties, can remain largely intact if properly treated [6]. Organ specific tissue may be harvested from allogenic or xenogeneic origin and treated to remove cells and DNA fragments to avoid graft rejection. By maintaining most of the structural integrity, construct properties can be very similar to the organ that is being treated for better integration of the graft. As the extracellular matrix is tissue and cell type specific, the use of these decellularized tissue may guide tissue ingrowth and improve the regeneration of tissue that currently shows slow regeneration (e.g. smooth muscle tissue) and reduce fibrosis by matching tissue properties.

Pre-implantation is another promising strategy to improve the performance of tissue engineered constructs. The constructs will be partially remodeled and vascularized at a non-functional location, which may improve graft acceptance when implanted to treat a defect. Different locations for pre-implantation have

been evaluated, including the omentum, peritoneum and subcutis [7-9]. A major drawback of pre-implantation is the need for an additional implantation and graft harvest procedure. For adequate tissue remodeling and vascular formation, several weeks are required. While this may be possible for several applications such as urethra reconstruction, it is less suitable for ureteral reconstruction. The nature of ureteral damage is usually acute and requires immediate surgery. Nevertheless, for severe and long ureteral defects a two-stage repair using a percutaneous nephrostomy catheter to drain urine between stages is a standard treatment option [10]. This time could be used for pre-implantation. However, investigating this method is complicated in animals due to their mobility which may lead to early elimination of the nephrostomy catheter. Current pre-clinical data suggests that pre-implantation may greatly improve the regenerative and functional outcome. Therefore, it should be considered for treatments that allow several weeks before intervention or when a two-stage procedure is indicated.

The addition of cells to tissue engineered grafts is still a topic of debate. Many individual studies report positive effects, but a recent systematic review shows that this is not the case for all outcome measures [11]. The addition of cells to tissue engineered constructs is thought to improve tissue regeneration by excreting cytokines and stimulating tissue development and is especially important for defects > 1.0 cm [12]. Stem cells are of great interest as they are readily available from autologous sources like the bone marrow or adipose tissue. These cells can differentiate to multiple cell types, including smooth muscle cells that are important for functionality of most urogenital organs. Similarly to pre-implantation, the use of cells may not be viable for all interventions as the isolation and expansion of the cells requires several weeks and an additional invasive procedure is required to harvest the cells. Even though the use of cells complicates the procedure, current results are promising and therefore the use of these cells should be further investigated.

When evaluating regeneration outcome, most studies only report qualitative data. One or more independent researchers usually perform histological scoring and a single figure is added to support these results. This method of analysis is subject to reporting bias as only one section of the regenerating tissue is shown. Therefore, reporting of qualitative data should also be considered when showing regenerating tissue. In chapter 6 and 7 of this thesis it was attempted to quantify muscle regeneration to get an objective measurement of tissue remodeling and reduce bias. In future studies, more sections should be evaluated in such a manner, preferably at multiple levels throughout the construct to improve appreciation of 3D tissue regeneration.

Currently, most investigators use constructs that are engineered in their own laboratories before application in an animal model. Whether these can be clinically implemented can be doubtful and may also explain the dissimilarity between construct used in pre-clinical and clinical studies. For successful clinical translation, it is important that these devices can be produced in GMP facilities according to ISO 13485 standards [13]. It is important to take these directives into account when designing the constructs for pre-clinical studies and preferably they should be produced under these conditions. This avoids repetitive evaluation when the production process cannot be translated from the lab to these facilities. Another important consideration is sterilization of the devices. Currently, several options are available, including autoclavation, gamma irradiation and ethylene oxide gassing. Depending on the material, not every option will be possible as it may influence the intrinsic scaffold/ material properties or even degrade the product [14-16]. Collaboration with industry partners that can help to design the production process to meet the regulatory requirements is crucial here.

When taking all these suggestions into consideration, significant steps can be taken towards clinical application of tissue engineered medical devices.

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Samenvatting

De behandeling van grote defecten aan het urogenitale stelsel blijft een uitdaging waarbij vaak donor weefsel zoals een huidflap, wangslijmvlies of darmweefsel nodig is. Ondanks dat er vaak een behandeling mogelijk is, is er een grote kans op bijwerkingen zoals (blijvende) pijn na het weghalen van het donorweefsel, metabole stoornissen of herhaling van het probleem. Het doel van dit proefschrift was het ontwikkelen van kunstweefsels als alternatief voor lichaamseigen donor weefsel. In de jaren zeventig begon het gebruik van biomaterialen toe te nemen in de reconstructieve geneeskunde. Toen het vakgebied in de jaren negentig snel begon te groeien werd de naam "tissue engineering" geïntroduceerd [1]. Het doel van dit snelgroeiende vakgebied is het vervangen of repareren van beschadigde organen en weefsels [2]. De vervaardigde constructen geven tijdelijke steun aan de beschadigde organen en weefsels en worden geleidelijke door het lichaam afgebroken en vervangen door lichaamseigen weefsel. De constructen kunnen specifiek ontworpen worden om aan de eigenschappen van het te repareren weefsel na te bootsen. Denk hierbij aan de mechanische eigenschappen en de snelheid waarmee het kunstweefsel wordt afgebroken. In de loop van de jaren zijn er veel pogingen gedaan om deze techniek naar de kliniek te brengen. Desalniettemin blijft ondanks het gebruik van verschillende biomaterialen, cellen en groeifactoren het gebruik van deze constructen binnen de urologie gelimiteerd. Alleen in gevallen waarbij de normale behandeling geen baat heeft, wordt tissue engineering overwogen [3-5]. In het eerste deel van dit proefschrift wordt de huidige stand van zaken besproken omtrent het gebruik van tissue engineering bij ureter (urineleider) en urethra (plasbuis) defecten. In het tweede deel wordt de ontwikkeling van nieuwe constructen op basis van collageen bestudeerd.

Een (systematisch) overzicht van de literatuur

In het eerste deel van dit proefschrift wordt een overzicht gegeven van ureter tissue engineering in dierproeven. Daarna volgt een systematisch overzicht van urethra tissue engineering in dierproeven en klinische studies.

Hoofdstuk 2 beschrijft de eerste pogingen om met tissue engineering de ureter te repareren. Hierbij werden vooral grote dieren zoals honden en varkens gebruikt. In deze modellen werd een groot defect gemaakt dat vervolgens behandeld werd met afbreekbaar collageen en darmweefsel waar de cellen uit werden gehaald of Gore-Tex, een niet afbreekbaar materiaal. In de meeste studies werden er aan deze constructen geen cellen toegevoegd. Ondanks dat weefsel herstel werd waargenomen op celniveau waren er veel bijwerkingen zoals excessieve bindweefselvorming dat voor vernauwing van de ureter en nier schade zorgde. Uit dit overzicht werd geconcludeerd dat de ureter mogelijk door middel van tissue engineering behandeld kan worden, maar dat er nieuwe technologie noodzakelijk is om bijwerkingen te voorkomen.

In **hoofdstuk 3** worden de meest recente studies naar ureter tissue engineering beschreven. Hierin was grote verandering in de aanpak waar te nemen. Er werden vaker cellen aangebracht en er werden meer natuurlijk materialen zoals collageen of gedecellulariseerde weefsels toegepast. Het gebruik van stamcellen nam ook toe en gaf veelbelovende resultaten in vergelijking tot volwassen cellen. Daarnaast werd een nieuwe operatietechniek geïntroduceerd waarbij de constructen tijdelijk elders in het lichaam geplaatst werden. Met deze zogenaamde pre-implantatie konden de constructen van bloedvaten voorzien worden en konden de stamcellen uitgroeien tot volwassen cellen. Opvallend was het kleine aantal nieuwe publicaties. Dit geeft weer dat het ontwikkelen van constructen om de ureter te behandelen erg uitdagend is.

Een interessante observatie is het gebrek aan klinische translatie, zelfs bij de urethra, het meest bestudeerde orgaan van het urogenitale stelsel. Om te onderzoeken waarom de klinische translatie beperkt is werd in **hoofdstuk 4** een systematisch overzicht van urethra tissue engineering uitgevoerd. Op deze manier werd een compleet overzicht gemaakt van alle gepubliceerde studies en kon de effectiviteit van verschillende behandelstrategieën met elkaar vergeleken worden. Opvallend was dat het gebruik van cellen in dierproeven tot een significant lager aantal bijwerkingen zorgt, terwijl dit effect in klinische studies niet waargenomen kon worden. Het gebruik van verschillende soorten biomaterialen of verschillende diermodellen had geen significant effect op het ontwikkelen van bijwerkingen, functionaliteit of de studietijd volbrengen. Uit analyse van de kwaliteit van de studies werd duidelijk dat het ontwerp van de studies verbeterd moet worden om tot klinische translatie te komen. Daarnaast dienen er betere controlegroepen gebruikt te worden en moet blindering en randomisatie toegepast worden. Als laatste is het belangrijk om de uitkomst van elk individueel dier te rapporteren.

Nieuwe hybride biomaterialen in urogenitale tissue engineering

In het tweede deel wordt de *in vivo* en *in vitro* ontwikkeling en evaluatie van nieuwe tubulaire en platte (hybride) collageen constructen besproken. In **hoofdstuk 5** wordt het effect van mechanische belasting op de groei en ontwikkeling van varkensblaas cellen bestudeerd. Deze cellen werden geïsoleerd uit de blaas van een varken en vervolgens op collageen matjes gekweekt. In een speciale bioreactor werd een mechanische rek op de matjes aangebracht om het vullen en legen van de blaas te simuleren. Deze rek zorgde voor een toename van de celgroei en zorgde voor het gelijk richten van de cellen. Op moleculair niveau konden geen verschillen worden waargenomen. Op basis van deze resultaten werd geconcludeerd dat het toepassen van mechanische stress tijdens het kweken van de cellen op collageen constructen mogelijk kan zorgen voor verbeterde ingroei van spierweefsel.

Naast de ingroei van spierweefsel is de vorming van nieuwe bloedvaten erg belangrijk om weefseldood te voorkomen. In **hoofdstuk 6** werden meerdere kleinere platte collageen constructen in de blaas van een varken getransplanteerd. Door meerdere kleine constructen te gebruiken kon hetzelfde oppervlak gerepareerd worden als met een groter construct, maar bleef de afstand die bloedvaten in moesten groeien beperkt. Om de bloedvat groei verder te stimuleren werden groeifactoren aan de constructen gebonden. Het gebruik van meerdere platte collageen constructen bleek een optie te zijn om het blaasoppervlak te vergroten. Het gebruik van groeifactoren leek geen effect te hebben op het weefselherstel. Een mogelijke oorzaak hiervan was de methode van binding aan

het construct. Doordat deze relatief zwak was kan het zijn dat de groeifactoren door urine uit het construct gewassen werd.

Om de ureter te repareren zijn tubulaire constructen nodig. In een pilotstudie in varkens (beschreven in **hoofdstuk 4)** werd duidelijk dat collageen alleen niet sterk genoeg is om gebruikt te worden voor behandeling van ureter defecten. Om de kracht van de constructen te versterken werd in **hoofdstuk 7** een afbreekbaar polymeer (Vicryl) toegevoegd. Daarnaast werden wederom groeifactoren aangebracht in een poging bloedvat ingroei te verbeteren. Het gebruik van Vicryl verbeterde de mechanische eigenschappen van de constructen en zorgde dat deze intact bleven. Door het gebruik van synthetische materialen trad er echter een grotere immuunreactie op waardoor de ureter sterk vernauwde en er nier schade optrad. Wederom werd er geen effect gezien bij het gebruik van groei factoren. Deze resultaten lieten zien dat er optimalisatie nodig is voor het gebruik van groei factoren en dat lokaal de immuunrespons gedempt moet worden.

In de voorgaande hoofdstukken werd het varken als diermodel gebruik. Ondanks de veelbelovende resultaten werden complicaties waargenomen die waarschijnlijk met het diermodel te maken hebben gehad. Zo werden er om praktische redenen jonge dieren gebruikt. Omdat jonge varkens erg snel groeien kan dit effect gehad hebben op de wondgenezing en hebben gezorgd voor de sterke bindweefselvorming vernauwing van de ureter. In **hoofdstuk 8** beschrijven we onze eerste ervaringen met een volwassen diermodel, de geit. Er werd voor geiten gekozen vanwege de grote gelijkenissen in gewicht en grootte met volwassen mensen en jonge varkens. Daarnaast bewegen geiten veel meer dan varkens. De operaties verliepen zonder problemen, maar bij het onderzoek na drie maanden bleek dat bij het plaatsen van de stent het nierkapsel doorgeprikt was. Om deze complicatie te voorkomen bleek het noodzakelijk te zijn om de stents met contrastvloeistof en röntgendoorlichting te plaatsen. Hieruit bleek dat het belangrijk is om ook bij dierproeven dezelfde voorzorgmaatregelen te nemen als bij mensen om onnodig lijden van de dieren te voorkomen.

In de laatste studie (**hoofdstuk 9)** van dit proefschrift worden de resultaten van de geiten studie besproken. Wederom werd gebruik gemaakt van collageen-Vicryl constructen zoals in de varkensstudie (**hoofdstuk 7).** In plaats van groeifactoren werd dit keer echter gebruik gemaakt van pre-implantatie. Door de constructen eerst een maand onder de huid te plaatsen konden bloedvaten ingroeien en werd het construct al gedeeltelijk omgevormd tot lichaamseigen weefsel. Het resultaat was een weefsel dat mechanische eigenschappen had die gelijkwaardig waren aan het ureter weefsel. Dit in tegenstelling tot de hybride constructen die veel stijver waren. Na pre-implantatie was de integratie in de ureter beter. Op celniveau werden er echter weinig verschillen waargenomen. De spiercellen groeiden even ver in beide type constructen. Bij de nieren waren wel grote verschillen waar te nemen. Waar bij het gebruik van hybride constructen was verbindweefseling, ontsteking en verandering van vorm waar te nemen was dit na pre-implantatie grotendeels normaal. Uit deze studie bleek het belang van het gebruik van constructen met mechanische eigenschappen die dicht bij het te behandelen weefsel liggen. Pre-implantatie lijkt een veelbelovende techniek om dit te bewerkstelligen.

Conclusie

In dit proefschrift wordt de huidige stand van zaken op het gebied van urogenitale tissue engineering beschreven doormiddel van (systematische) overzichten van de literatuur. Nieuwe trends op het gebied van tissue engineering konden worden geïdentificeerd, alsmede redenen voor een beperkte klinische translatie. Belangrijke punten hierbij is het ontwerp van de dierproeven, specifiek het gebruik van de juiste controle groepen, blindering en randomisatie. Daarnaast beschrijft dit proefschrift de ontwikkeling van (hybride) collageen constructen voor het vergroten van de blaascapaciteit en het repareren van grote ureter defecten. Om de uitkomst te verbeteren is gebruik gemaakt van cellen, groeifactoren en pre-implantatie. Van deze technieken lijkt pre-implantatie het meest veelbelovend en misschien zelfs noodzakelijk voor het gebruik in de kliniek.

Toekomstvisie

Klinische translatie van de kennis die opgedaan is tijdens dierproeven en laboratoriumonderzoek is een cruciale stap die genomen moet worden in het vakgebied van tissue engineering. Uit hoofdstuk 4 is duidelijk geworden dat het ontwerp van dierproeven verbeterd moet worden om deze klinische translatie te bewerkstelligen. Het belangrijkste aandachtspunt is het gebruik van de juiste controle groepen, zoals het gebruik van een huid flap, wangslijmvlies of darmweefsel. Een andere opmerkelijke bevinding was het materiaal dat gebruikt wordt in de klinische studies. In tegenstelling tot de dierproeven, waar een grote variatie aan materialen wordt gebruikt, werden er voornamelijk gedecellulariseerde weefsels zonder toegevoegde cellen toegepast. Daarnaast is het aan te bevelen om de duur dat de dieren in het experiment zitten te verlengen. De meeste dierproeven duren momenteel drie tot zes maanden, terwijl patiënten nog vele jaren zullen leven na de operatie. Het is daarom aan te bevelen om ook langere tijdspunten (> 12 maanden) te onderzoeken. Hierdoor zullen niet alleen de korte termijn complicaties bekend worden, maar ook complicaties die pas later optreden. Daarnaast geeft dit een beter beeld van de weefselgenezing over de jaren.

Er wordt momenteel veel energie gestoken in het optimaliseren van biomaterialen zoals collageen. Zo worden verschillende concentraties, combinaties van materialen en groeibevorderende eiwitten getest en gecombineerd. Hoewel het met tissue engineering mogelijk is om deze condities uit te proberen is het momenteel door de complexiteit van lichaamsweefsel onmogelijk om deze weefsels goed na te maken in het laboratorium. Gedecellulariseerde materialen kunnen een goede stap zijn aangezien de structuur en weefseleigenschappen grotendeels intact kunnen blijven [6]. Een mogelijkheid is het gebruik van orgaan specifiek weefsel van allogene of xenogene oorsprong dat behandeld is om alle cellen en DNA restanten te verwijderen. Doordat de weefselstructuur en weefseleigenschappen grotendeels intact blijven lijkt het weefsel veel op het te

behandelen weefsel. Dit kan zorgen voor een betere integratie van het construct. De structuur van het gedecellulariseerde weefsel is orgaan en celtype specifiek. Hierdoor kunnen deze weefsels de ingroei van belangrijk functioneel weefsel, zoals spiercellen, bevorderen. Daarnaast kan het tot een vermindering van bindweefselvorming zorgen.

Een andere veelbelovende strategie is het pre-implanteren van een tissue engineered construct. Doordat de constructen gedurende de pre-implantatie periode deels vervangen worden door lichaamseigen weefsel en er bloedvat ingroei plaats kan vinden kunnen de eigenschappen verbeterd worden. Er zijn verschillende plekken onderzocht waar het weefsel tijdelijk geplaatst kan worden, bijvoorbeeld in het omentum, peritoneum en de subcutis [7-9]. Een groot nadeel van pre-implantatie is de lange tijd die hiervoor nodig is. Om bloedvat en weefsel ingroei te krijgen zijn meerdere weken nodig. Dit is voor veel behandelingen geen probleem aan gezien deze vaak gepland kunnen worden. Voor de ureter kan dit lastig zijn, aangezien ureter trauma vaak acuut is, waardoor direct behandeling nodig is. Desalniettemin is er bij ernstig trauma vaak een tweede procedure nodig waarbij de eerste alleen dient ter stabilisatie. Er wordt dan tijdelijk een katheter geplaatst waardoor er geen urine door de ureter loopt [10]. De tijd die nodig is om te stabiliseren zou gebruikt kunnen worden voor de pre-implantatie. Het onderzoeken van deze methode is echter gecompliceerd aangezien dieren erg mobiel zijn en de katheter zullen proberen te verwijderen. De huidige dierproeven suggereren dat pre-implantatie een grote verbetering kan geven bij het weefsel herstel en de functionaliteit van het urine systeem. Het is daarom aan te raden om pre-implantatie te overwegen als de operatieplanning dit toe laat.

Het toevoegen van cellen is een controversieel onderwerp waar veel discussie over plaats vindt. Veel onderzoeken rapporteren positieve resultaten. Een recente systematische vergelijking laat echter zien dat dit niet voor alle uitkomsten geldt [11]. Cellen worden toegevoegd vanwege de hypothese dat deze groeibevorderende eigenschappen hebben, zoals het uitscheiden van eiwitten en het sturen van de uitgroei van stamcellen. Dit is voornamelijk belangrijk bij defecten > 1.0 cm [12]. Stamcellen worden veel onderzocht omdat deze in grote getalen te verkrijgen zijn van de patiënt zelf, bijvoorbeeld uit het beenmerg of vetweefsel. Daarnaast kunnen deze cellen uitgroeien tot een groot aantal volwassen cellen, waaronder spiercellen die belangrijk zijn voor de werking van het urogenitale stelsel. Het opkweken van deze cellen is echter een tijds- en arbeidsintensief proces, waardoor dit niet altijd mogelijk is bij de behandelingen van ureter defecten. Daarnaast is een invasieve handeling nodig om de cellen te verkrijgen. Desalniettemin zijn de resultaten veelbelovend en zal het gebruik van stamcellen verder onderzocht moeten worden.

Momenteel wordt de uitkomst van het weefselherstel voornamelijk kwalitatief onderzocht, waarbij een of meerdere onafhankelijke onderzoekers een oordeel geven. Deze methode heeft een grote kans op vooroordelen bij de beoordeling aangezien maar een klein deel van het weefsel bekeken wordt. Het toepassen van kwantitatieve beoordeling kan deze vooroordelen verminderen aangezien het gehele weefsel automatisch onderzocht kan worden. In hoofdstuk 6 en 7 van dit proefschrift is getracht de spierweefsel ingroei kwantitatief te beoordelen om zo een onbevooroordeeld oordeel over de weefselregeneratie te kunnen geven. In toekomstige studies kan deze methode gebruikt worden om een groter aantal niveaus van het weefsel te beoordelen en zelfs driedimensionale reconstructies te maken.

Momenteel worden de meeste constructen die gebruikt worden in dierproeven gefabriceerd in het laboratorium van de onderzoekers. Het is dan ook maar de vraag of deze constructen ooit gebruikt zullen worden voor klinische toepassingen. Voordat deze materialen naar de kliniek gebracht kunnen worden is het belangrijk dat deze onder GMP condities en volgens ISO standaarden gefabriceerd worden [13]. Het is niet vanzelfsprekend dat een construct dat in het laboratorium gemaakt kan worden ook onder deze omstandigheden vervaardigd kan worden. Het is daarom aan te bevelen dat er al rekening gehouden wordt bij het ontwerpen van de constructen dat het in de toekomst onder bepaalde omstandigheden gemaakt moet kunnen worden. Sterilisatie is een belangrijk voorbeeld hiervan. Veel materialen die gebruikt worden in dierproeven worden gedesinfecteerd met ethanol, terwijl ze gesteriliseerd moeten worden voor klinisch gebruik. Dit kan met behulp van autoclaveren, bestraling en het gebruik van bepaalde gassen. Echter, niet alle materialen zijn geschikt om gesteriliseerd te worden met deze opties aangezien ze van invloed kunnen zijn op de eigenschappen van de constructen [14-16]. Dit kan uiteindelijk zorgen van discrepanties tussen dierproefresultaten en eerste klinische proeven. Samenwerking met partners uit de industrie is belangrijk om te zorgen dat het productieproces mogelijk is en dat er voldaan kan worden aan de regelgeving.

Wanneer de bovenstaande aanbevelingen in acht genomen worden kunnen er grote stappen gezet worden om tissue engineering naar de kliniek te brengen.

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Chapter 11

Dankwoord, list of publications, PhD portfolio and Curriculum Vitae

Dankwoord

Mijn proefschrift is klaar! Graag wil ik terugkijken op de mooie tijd die ik heb gehad vol met productieve samenwerkingen, veel gezelligheid en nieuwe vriendschappen. Ik wil iedereen bedanken die de totstandkoming van dit proefschrift mogelijk heeft gemaakt.

Prof. Dr. W.F.J. Feitz, beste Wout. Bedankt voor het vertrouwen dat je me hebt gegeven toen ik halverwege mijn afstuderen vroeg of er een mogelijkheid was om na mijn afstuderen te blijven om te promoveren. Je positieve instelling en manier van aanpakken zijn een grote inspiratie voor mij. “Gewoon even regelen” schiet me altijd te binnen als ik een berg voor me zie in plaats van een heuveltje. Bedankt voor alle mogelijkheden, leermomenten en steun.

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Beste Tissue Engineers, met jullie heb ik het meest samengewerkt en daar heb ik goede herinneringen aan over gehouden. Marije, ik wil jou bedanken voor de leuke tijd die we samen hebben doorgebracht om onze studies tot een succes te brengen. Van de vele uren in de clean room en aan de operatietafel in het CDL, tot de vele ritjes naar de boerderij en de kleinere uurtjes in de Aesculaaf. We waren een goed team! Peter, het is maar goed dat we niet naast elkaar zaten in het lab, anders was ik waarschijnlijk nog met mijn eerste artikel bezig! Je bent een goede vriend geworden en hebt zeker bijgedragen aan de mooie tijd die ik op het lab gehad heb. Ook ben ik je dankbaar voor de mooie mechanische data in mijn manuscripten. Ik hoop dat we nog vaak een “klein” biertje in de Aesculaaf kunnen drinken of een uitstapje naar Kranenburg kunnen maken. Dorien, zonder jou is het lab niet hetzelfde. Je stond altijd voor me klaar als er weer eens een experiment niet wilde lukken en we hebben veel kunnen lachen tijdens de koffiepauzes. Bedankt! Silvia en Alejandra, ik wil jullie bedanken voor de goede gesprekken en de leuke momenten die we gehad hebben. Ik hoop jullie nog vaak tegen te komen. Bronte en Rayna, bedankt voor de mooie tijd. Lisa en Marcel, ik ben dankbaar voor jullie bijdrage tijdens jullie stages.

Daarnaast ben ik dankbaar dat ik het lab heb mogen delen met een aantal geweldige collega’s. Gerald, Ola, Onno, Jasmijn, Cindy, Cindy (Rönnau), Sebastian, Mirjam, Tilly, Kees, Jeanette, Anglita, Vicky, Elze, Marion, Nicolien en Renate, ontzettend bedankt voor de fijne tijd op het lab. Ook wil ik Boy, Allert, Dick, Hans, Rianne, Caroline, Tom, Siebren en Max, de artsen en arts-onderzoekers die (soms) op het lab te vinden waren bedanken voor alle onvergetelijke momenten. Boy en Allert, met 9gag, de speld, flauwe woordgrappen en regelmatig een biertje ben ik blij dat ik jullie heb leren kennen. Beste Jack, bedankt dat ik bij jou op het lab mijn onderzoek heb kunnen doen. Je hebt een leuke club mensen bij elkaar weten te krijgen en houden!

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List of publications

1. **De Jonge, P.**, Sloff, M., Janke, HP., Kortmann, B., de Gier, R., Geutjes, P., Oosterwijk, E., Feitz, W. "Ureteral reconstruction in goats using tissue engineered templates and subcutaneous pre-implantation." *Tissue Eng Part A 2017, doi:10.1089/ten.TEA.2017.0347*
2. Versteegden, L.*, **de Jonge, P.***, IntHout, J., van Kuppevelt, T., Oosterwijk, E., Feitz, W., de Vries, R., Daamen, W. "Tissue engineering of the urethra: a systematic review and meta-analysis of pre-clinical and clinical studies." *Eur Urol. 2017, doi:10.1016/j.eururo.2017.03.026* *Equal contribution.
3. **De Jonge, P.**, Sloff, M., Janke, HP., Kortmann, B., de Gier, R., Geutjes, P., Oosterwijk, E., Feitz, W. "Clinical protocol levels are required in laboratory animal surgery when using medical devices: experiences with ureteral replacement surgery in goats." *Lab Anim. 2017, doi:10.1177/0023677217696520.*
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8. **De Jonge, P.**, Simaioforidis, V., Geutjes, P., Oosterwijk, E., Feitz, W. "Recent advances in ureteral tissue engineering." *Curr Urol Rep. 2015, doi:10.1007/ s11934-0140465-7.*
9. Simaioforidis, V.*, **de Jonge, P.***, Sloff, M., Oosterwijk, E., Geutjes, P., Feitz, W. "Ureteral tissue engineering: where are we and how to proceed?" *Tissue Eng Part B Rev. 2013, doi:10.1089/ten.teb.2012.0737.* *Equal contribution.

PhD portfolio

Name PhD candidate: P.K.J.D. de Jonge	PhD period: *01-07-2013 – 31-12-2016*
Department: Urology	*Promotor(s): Prof. W.F.J. Feitz*
Graduate School: RIMLS	*Co-promotor(s): Dr. E. Oosterwijk, Dr. P.J. Geutjes*

		Year(s)	ECTS
	TRAINING ACTIVITIES		
a)	***Courses & Workshops***		
-	Introduction day Radboudumc	2013	0.5
-	Graduate School specific introductory course	2013	2.0
-	Academic Writing	2014	3.0
-	Refresher course in Statistics	2014	1.0
-	Basic Histopathology course	2014	0.75
-	Presentation skills	2015	1.5
-	Advanced Conversation	2015	1.5
-	The art of presenting science	2015	1.5
-	Entrepreneurship and innovation for PhD students	2016	3.0
b)	***Seminars & lectures***		
-	Technical for a (5x)	2013-2016	1.0
-	Seminars (6x)	2013-2014	0.6
-	Noon spotlight	2013	0.1
-	Radboud Research Rounds (9x)	2014-2016	0.9
c)	***Symposia & congresses***		
-	Radboud Frontiers	2013-2014	2.0
-	PhD retreat #,#,*	2014-2016	2.5
-	NVvTG annual conference *	2013	0.5
-	REFORM symposium	2013	0.1
-	IRB Barcelona PhD retreat #	2013	1.0
-	NBTE annual conference #,*,*	2013-2016	2.25
-	Health Valley: Pecha Kucha *	2014	0.25
-	EAU annual conference *,*	2014-2015	3.0
-	TERMIS annual conference #,#,*	2014,2016	3.5
-	ESUR-SBUR annual conference #,#,#,#	2015	1.75
d)	***Other***		
-	Journal Club (8x)	2013-2016	2.0
-	Organization NVvTG annual conference	2014-2015	2.0
	TEACHING ACTIVITIES		
e)	***Lecturing***		
-	*na*		
	f) Supervision of internships / other		
-	Graduation Internship HLO, HAN	2015	2.0
-	Master internship Technical Medicine, University of Twente	2015	1.0
	TOTAL		**38.8**

Oral and poster presentations are indicated with a * and # after the name of the activity, respectively.

Curriculum Vitae

Paul de Jonge werd op 5 september 1987 geboren te Gouda. In 2005 haalde hij zijn VWO diploma aan het Twents Carmel College de Thij in Oldenzaal. Direct hierna is hij begonnen aan de bacheloropleiding Technische Geneeskunde aan de Universiteit Twente in Enschede, gevolgd door de masteropleiding Technical Medicine. Tijdens zijn master heeft Paul zich gespecialiseerd in reconstructieve geneeskunde. Bij zijn eerste masterstage op de afdeling urologie (Radboudumc, Nijmegen) kwam Paul in aanraking met tissue engineering. Na een aantal uitstapjes bij de medische immunologie (Radboudumc, Nijmegen), de vaatchirurgie (MST, Enschede) en biomedical engineering (Case Western Reserve University, Cleveland, OH, USA) keerde hij terug naar de afdeling urologie van het Radboudumc voor zijn afstudeeronderzoek. Zijn onderzoek naar tubulaire collageen constructen om urineleiders in varkens te repareren is de basis van zijn promotieonderzoek geweest.

In juli 2013 is Paul gestart met promotieonderzoek bij de afdeling urologie van het Radboudumc. Dit onderzoek is onder begeleiding van Prof. dr. W.F.J. Feitz, dr. E. Oosterwijk en dr. P.J. Geutjes uitgevoerd. De resultaten staan beschreven in dit proefschrift, zijn gepresenteerd op (inter)nationale congressen en gepubliceerd in internationale tijdschriften. Tijdens zijn promotieonderzoek is hij actief geweest bij de Nederlandse Vereniging voor Technische Geneeskunde (NVvTG), waar hij medeverantwoordelijk was voor de organisatie van het 5^{e} jaarlijkse NVvTG congres.

Sinds juli 2017 is Paul postdoctoraal onderzoeker bij de immunotherapie onderzoeksgroep van het laboratorium van hematologie in het Radboudumc onder leiding van dr. H. Dolstra. Het project is gericht op de klinische toepassing van natural killer cel therapie bij ovariumkanker.