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Comparison of poly(L-lactide-co-ε-caprolactone) and poly(trimethylene carbonate) membranes for urethral regeneration: an *in vitro* and *in vivo* study

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Abstract

Urethral defects are normally reconstructed using patient's own genital tissue; however, in severe cases, additional grafts are needed. We studied the suitability of poly(L-lactide-co-ε-caprolactone) (PLCL) and poly(trimethylene carbonate) (PTMC) membranes for urethral reconstruction *in vivo*. Further, the compatibility of the materials was evaluated *in vitro* with human urothelial cells (hUC). The attachment and viability of hUCs and the expression of different urothelial cell markers (Cytokeratin 7, 8, 19 and Uroplakin Ia, Ib and III) were studied after *in vitro* cell culture on PLCL and PTMC. For the *in vivo* study, 32 rabbits were divided into the PLCL (n=15), PTMC (n=15) and control or sham surgery (n=2) groups. An oval urethral defect 1×2 cm in size was surgically excised and replaced with a PLCL or a PTMC membrane or urethral mucosa in sham surgery group. The rabbits were followed for 2, 4 and 16 weeks. After the follow-up, urethrography was performed to check the patency of the urethra. The defect area was excised for histological examination, where the epithelial integrity and structure, inflammation and fibrosis were observed.

There was no notable difference on hUCs attachment on PLCL and PTMC membranes after 1 d of cell seeding, further, the majority of hUCs were viable and maintained their urothelial phenotype on both biomaterials. Postoperatively, animals recovered well, and no severe strictures were discovered by urethrography. In histological examination, the urothelial integrity and structure developed towards a normal urothelium with only mild signs of fibrosis or inflammation**.**

According to these results, PLCL and PTMC are both suitable for reconstructing urethral defects. There were no explicit differences between the PLCL and PTMC membranes. However, PTMC membranes were more flexible, easier to suture and shape and developed significant epithelial integrity.

Keywords: poly(L-lactide-co-ε-caprolactone), poly(trimethylene carbonate), urethral defects, urethral tissue engineering, urothelial cell

1. Introduction

Urethral defects due to congenital causes, trauma or infection are fairly common. For instance the prevalence of hypospadia, which is a common congenital anomaly, has increased during the last decades now being approximately 1/250 to 1/300 live births.[1] Small urethral defects are traditionally reconstructed using the patient's own genital tissue. However, reconstruction of large urethral defects requires additional grafts, such as buccal mucosa. Nevertheless, complications, such as urethral strictures, diverticulas, and fistula formation, are relatively common in these operations. Further, using the patient's own tissue as a graft material leads to donor site morbidity.[2,3] Thus there is a clear clinical need for new reconstruction techniques of urethral defects.

Tissue engineering could provide a novel method to overcome problems associated with traditional reconstructive surgery. Several natural tissue grafts, such as bladder acellular matrix (BAMG) and collagen and small intestine mucosa (SIS) with and without cells, have previously been studied for urethral reconstruction. For instance, Orabi *et al.* studied the BAMG seeded with urothelial and smooth muscle cells for urethral reconstruction in an *in vivo* beagle model with promising results. They compared the cell-seeded BAMG with the non-cellular BAMG graft and observed that, in the non-cellular group, the number of urethral strictures and fistulas was remarkably higher.[4] The disadvantage of natural biomaterials is the high batch-to-batch variation, and large-scale manufacturing and the modification of mechanical properties are difficult. Thus, the development of novel graft materials for urethral reconstruction is essential to develop new treatment options for remedying severe urethral defects.[2,3,5,6]

The selection of an appropriate biomaterial for the application is crucial, and the biomaterial for urethral reconstruction should meet the requirements of being biocompatible, nontoxic, biodegradable without disadvantageous tissue reactions and able to promote urothelial tissue regeneration. Furthermore, for urethral reconstruction, the biomaterial should be elastic and flexible and should mimic the basement membrane of the urothelium, generating a suitable matrix

for urothelial cells to attach and proliferate. Additionally, the biomaterial should be suturable and easily molded into a tubular structure.[2,6]

Aliphatic poly(α-esters), such as polyglycolide (PGA), polylactide (PLA), polycaprolactone (PCL) and their copolymers, are the most commonly studied synthetic biomaterials for tissue engineering, and they have also been studied in urological applications with promising results.[7,8,9] Tubular PGA:poly(lactide-co-glycolide acid) (PLGA) scaffolds seeded with urothelial and smooth muscle cells were used to reconstruct urethras for 5 boys suffering from severe urethral defects with favorable results. After the operation, a narrowing of the urethra developed for one patient, but it was repaired with a surgical incision. After a 6-year follow-up, no strictures or diverticula were detected, and the urethral histology was normal after 3 months.[8]

Furthermore, PLGA scaffolds were also used to reconstruct *de novo* bladders for children suffering from neurogenic bladders; however, the results of this study were not positive. In this study, PLGA scaffolds seeded with urothelial cells and smooth muscle cells were used to reconstruct *de novo* bladders for 10 children. Severe adverse effects, either bowel obstruction or bladder rupture, were detected in 4 patients.[9] Furthermore, Pariente *et al*. have demonstrated excellent biocompatibility of PGA, poly-L-lactic acid (PLLA) and PLGA when cultured with urothelial cells *in vitro*.[7] Although the aliphatic poly(α-esters) are considered as potential biomaterials for urological applications, intensive research is required before tissue engineered urethral grafts can be used as an everyday treatment method for urethral defects. In particular, the development of optimal scaffold material and design is essential.

In our previous studies, we have shown that human urothelial cells attach, remain viable, and proliferate on poly(L-lactide-ε-caprolactone) (PLCL) membranes *in vitro*.[10,11] PLCL is a biocompatible copolymer of L-lactide and ε-caprolactone with variable mechanical properties depending on the monomer ratio. Increasing the ε-caprolactone content results in a more flexible and elastic polymer. PLCL degrades mainly via hydrolysis, although enzymes may also affect the

degradation at latter stages.[12,13] Furthermore, PLCL has been previously studied in other soft tissue engineering applications, such as vascular and esophageal tissue engineering, with encouraging results, and due to its excellent biocompatibility and elasticity, it is an interesting biomaterial for urethral reconstruction.[12,14]

Poly(trimethylene carbonate) (PTMC) is a benign, degradable, biocompatible polymer prepared from trimethylene carbonate that possesses good mechanical properties.[15,16] PTMC is glass-like at temperatures below approximately –15°C but is flexible at room temperature.[15] PTMC degrades via surface, not bulk, erosion as well as enzymatically without acidic end products *in vivo*.[17-20] PTMC has been studied in various tissue engineering applications, for instance for cardiomyocyte and Schwann cell cultivation, guided bone regeneration and abdominal surgery. Further, PTMC has been studied for vascular tissue engineering applications with good results.[15, 19] Due to its flexibility, biocompatibility and potential for soft tissue engineering applications, we considered it an interesting biomaterial for urothelial applications. Further, at least to our knowledge, PTMC has not been previously studied for urothelial tissue engineering.

Due to the unmet medical need of nonurological grafts, we tested PLCL and PTMC membranes for urothelial tissue engineering. The aim of this study was to compare the suitability and *in vivo* biocompatibility of PLCL and PTMC for urethral reconstruction in an *in vivo* rabbit model.

2. Materials and methods

2.1. Biomaterial membranes

The 70/30 poly(L-lactide-co-ε-caprolactone) surface-textured membranes were provided by Proxy Biomedical (Proxy Biomedical Ltd, Galway, Ireland). The PLCL membranes were manufactured by film molding, resulting in 200-µm thick membranes. Surface texturing of the films was accomplished with a 100 watt CO_2 laser micromachining device (Preco-Europe Inc.,

Canterbury, UK) giving a pitted surface texture. The samples were sterilized using gamma irradiation at 25 kGy.

PTMC was synthesized by ring polymerization of trimethylene carbonate (Boehringer Ingelheim, Ingelheim am Rhein, Germany) under a blanket of nitrogen at 150°C, using stannous octoate (Sigma Aldrich, St. Louis, USA) as a catalyst and water as an initiator. The polymer was melted using a compression molder (Fontijne laboratory press THB400, Vlaardingen, The Netherlands) at 160°C and up to 100 kN for one minute, followed by a two-step compression molding cycle. Membranes with a thickness of 250 µm were obtained, their molecular weight was approximately 275 kg/mol. The membranes were cut to appropriate size and packed in PET/ALU/PE peel pouches (Riverside medical packaging ltd., Derby UK), vacuum sealed and gamma irradiated at 25 kGy using a ^{60}Co source (Synergy Health, Ede, The Netherlands) for sterilization and cross-linking. The PTMC membranes were prepared in a similar manner as described in previous studies.[16,17]

The X-ray microtomography (µCT) images of PLCL and PTMC membranes were acquired by using commercial Zeiss Xradia MicroXCT-400 (Zeiss, Pleasanton, USA) system (Figure 1). PLCL and PTMC membranes were imaged respectively with the following parameters: 60 kV source voltage, 10 W tube power; 40 mm source-to-object distance; 8 mm object to image-receptor distance; 20x, 10x objective; 2 binning; 1600 projections; full 360° projection circle; and 5.0 s, 2.5 s exposure time. The three-dimensional image stacks were reconstructed using Zeiss Xradia XRMreconstructor software (8.1, Zeiss) resulting in 1.1 µm and 2.3 µm isotropic voxel sizes and the data was visualized in Zeiss Xradia TXM3DViewer (1.1.6, Zeiss).

2.2. In vitro cell culture

For this study, human urothelial tissue samples were isolated during a routine surgery from one pediatric patients in the Tampere University Hospital with the approval of the Ethics Committee of

Pirkanmaa Hospital District, Tampere, Finland (R071609). Further, the urothelial cells were isolated and expanded as previously described.[10,21] PLCL and PTMC membranes were attached to the cell culture devices (CellCrown48, Scaffdex, Tampere, Finland) and preincubated for 24 hours in urothelium medium containing EpiLife (Invitrogen, MA, US) supplemented with 1 % of EpiLife Defined Growth Supplement (EDGS, Invitrogen), 0.1 % of $CaCl_2$ (Invitrogen) and 0.35 % of antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin; Lonza, BioWhittaker, Verviers, Belgium).

2.3 In vitro attachment and viability

The cell attachment was verified by determining the DNA amount using CyQUANT Cell Proliferation Assay kit (Invitrogen). Briefly, 20 000 urothelial cells from one patient were seeded on to 3 parallel PLCL and PTMC membranes and cultured for 24 h. The cells were lysed with 0.1 % Triton-X-100 buffer (Sigma-Aldrich) and stored at -70 °C until analysis. The samples were thawed and 20 µl of each sample was mixed with 180 µl of working solution containing CyQUANT GR dye and lysis buffer. The fluorescence at 480/520 nm was measured with a multiplate reader (Victor 1420 Multilabel Counter; Wallac; Turku, Finland).

To verify the viability of human urothelial cells on PLCL and PTMC membranes, we used qualitative live/dead fluorescent staining. The urothelial cells from one patient, 30 000 cells/cm^2, were seeded on to two parallel membranes and the viability of urothelial cells was verified after 1 and 2 weeks of cell culture as described before.[10,11] Briefly, the cells were incubated at room temperature with a mixture of 0.25 µM calcein AM (green fluorescence, Molecular Probes, Waltham, USA) and 0.3 µM ethidium homodimer-1 (red fluorescence, EthD-1, Molecular Propes) for 30 minutes. A fluorescence microscope (Olympus, IX51S8F-2, camera DP71, Tokyo, Japan) was used to image viable cells (green fluorescence) and dead cells (red fluorescence).

2.4. Quantitative real-time PCR

The relative expression of urothelium marker genes was studied after 14 d of cell culturing on PLCL or PTMC with quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The cell culture polystyrene (PS) served as a control material. For the experiment 50 000 cells/cm^2 from one patient was seeded on to three parallel PLCL, PTMC or PS wells and cultured until analyses. First, the total RNA was isolated with Nucleospin kit reagent (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Thereafter, the RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Life Technologies). The expression of cytoceratin (CK) 7, CK8, CK 19, uroplakin (UP) Ia, UPIb and UPIII was analyzed. The expression data were normalized to the expression of housekeeping gene RPLP0 (large ribosomal protein P0). The sequences of primers (Oligomer Oy, Helsinki, Finland) and the accession numbers are presented in the Table1. The qRT-PCR mixture contained cDNA, forward and reverse primers and SYBR Green PCR Master Mix (Applied Biosystems). The reactions were conducted with AbiPrism 7000 Sequence Detection System (Applied Biosystems) with initial enzyme activation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 60 s. The previously described mathematical model was used to calculate the relative expression.[22]

2.5. In vivo experiment

The animal experiment was conducted under the license of the Board of Animal Experiments (ESLH-2009-06718/Ym-23), and the National Research Council's Guide for the Care and Use of Laboratory Animals was followed. Adult male New Zealand White rabbits (n=34, Harlan Laboratories, Netherlands) were housed in the Animal Laboratory of the University of Tampere throughout the study. The rabbits were divided into the following 3 reconstruction groups: 15 rabbits with a PLCL membrane, 15 rabbits with a PTMC membrane, and two rabbits serving as a control group with urethral mucosa for reconstruction.

First, the rabbits were weighed and then anesthetized using a combination of 0.3 mg/kg medetomidine (Domitor, Orion Inc., Espoo, Finland) and 0.3 mg/kg ketamine (Ketalar, Parke Davis Inc., Caringbah, NSW, Australia), which were given intramuscularly. Additionally, the prophylactic antibiotic 2.5 mg/kg enrofloxacin (Baytril vet 50 mg/ml, Bayer Animal Health GmbH, Leverkusen, Germany) was given intramuscularly prior to the surgery. The rabbits were catheterized using 8F catheters (Mediplast AB, Malmo, Sweden). An incision approximately 2 cm long was created in the rabbit skin from the inguinal to the penile region, and the urethral mucosa was exposed (Figure 2A). First, a 2×1-cm oval-shaped defect was created to the urothelial mucosa of the rabbit's anterior urethra. Holding sutures made of 6/0 nonabsorbable polypropylene (Premilene®, B. Braun Medical AS, Melsungen, Germany) were placed in every defect quarter and left in place after the operation as marking sutures. The defect site was replaced with the same sized on-lay PLCL or PTMC membrane (Figure 2B), which was tailored just before transplantation using surgical scissors. The biomaterial membranes were sutured to the free edges of the urothelial mucosa with bioabsorbable 6/0 poly(p-dioxanone) sutures (PDS II®, Ethicon Inc., New Jersey, US) and aligned with the catheter (Figure 2C). For control rabbits, we did a sham surgery, removed a similar patch of urethral mucosa and sutured it back as a graft to the defect area to investigate the inflammation caused by the operation and the absorbable suture. After the suturing, the skin wound was closed with an intracutaneous suture using absorbable 4/0 polyglactin 910 sutures (Vicryl®, Ethicon Inc., New Jersey, US), and the catheter was removed. The adequate analgesia was administered, and all the rabbits received 4 mg/kg carprofen (Norocarp vet, Norbrook Laboratories Ltd., Newry, Northern Ireland) and 0.05 mg/kg buprenorphine (Temgesic, Schering-Plough Europe Inc., Brussels, Belgium) subcutaneously during the operation. The 0.05 mg/kg buprenorphine (Schering-Plough Europe Inc.) was continued until 24 h after the operation, and the carprofen (Norbrook Laboratories Ltd.) was given daily 2 days after the operation. Pain medication was continued longer, if required. The rabbits in both the PLCL and PTMC groups were followed up for

2, 4 or 16 weeks, individually caged on an ad libitum diet. The sham surgery rabbits were followed for up to 2 or 4 weeks.

2.6. In vivo follow-up

After the follow-up, the animals were weighed, anesthetized as described in section 2.3 and subjected to urethrographic examination. The animals were catheterized, and the 8F catheter (Mediplast AB) was fixed with sutures distal from the defect area, which was palpated during catheterization. The urethrographic examination was performed by administering 180 mg/ml iohexol (Omnipaque, GE Healthcare AS, Oslo, Norway) by syringe as a radiocontrast agent via the catheter towards the bladder and by taking simultaneous X-ray pictures (Philips Oralix, Amsterdam, Holland) to detect severe strictures. Severe strictures block urine flow and thus prevent normal urination. After the examination, the animals were euthanized using 1 mg/kg intravenous pentobarbital (Mebunat, Orion Inc., Espoo, Finland). The defect area was then excised, cut perpendicular from the middle of the reconstructed urethra in order to get the defect centre to the histological analyses, and stored in 4 % paraformaldehyde (Sigma- Aldrich) until histolgical analyses.

2.7. Histology

Paraformaldehyde-fixed tissue samples from rabbit urethras were embedded in paraffin and stained with hematoxylin and eosin (H&E) (Reagena Oy, Finland) or Masson´s trichrome (Sigma-Aldrich) for microscopic examination. Epithelial integrity and structure were determined from the H&E-stained samples 2, 4 and 16 weeks after the operation. Epithelial integrity was categorized as discontinuous or continuous, whereas epithelial structure was categorized as no structure, monolayered or layered, i.e., stratified structure.[23] Edema and the presence of inflammatory cells in the H&E-stained samples were evaluated to discover

inflammation. Edema was scored from zero to three: 0 = none, 1 = mild, 2 = moderate and 3 = severe.[23] Similarly, the presence of inflammatory cells was scored from zero to three. Normal inflammatory cell appearance was scored as zero; less than 25 % of all cells was scored as one; 25-50 % was scored as two and over 50 % was scored as three.[24] The epithelial integrity and structure and inflammation-related parameters were examined at 40× magnification. Fibrosis was determined from Masson´s trichrome-stained samples and scored. A score of zero indicated no fibrosis, one indicated mild fibrosis (less than 25 %), two indicated moderate fibrosis (25-50 %) and three indicated severe fibrosis (more than 50 %).[25] The histological examination was performed without knowing the group to which the sample belonged.

2.8. Immunohistochemistry

Immunohistochemistry with the pancytokeratin marker was used to study the urothelial epithelium after 2, 4 and 16 weeks of follow-up. Briefly, the samples were fixed with 5 % paraformaldehyde and embedded in paraffin. The antigen retrieval was performed by microwaving the samples in 10 mM EDTA buffer (pH 9, Sigma-Aldrich), after which the samples were blocked in 3 % hydrogen peroxide (Sigma-Aldrich). The samples were incubated overnight in diluted primary antibody (1:100, AE1/AE3, Thermo Fisher Scientific, MA, US). On the following day, the secondary antibody (1:200, goat anti-mouse IgG, Thermo Fisher Scientific) was used to detect to the primary antibody.

2.9. Statistical analysis

Statistics were analyzed using IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA). Edema, inflammation cell appearance, epithelial structure, fibrosis and cell attachment on membranes were analyzed using the Mann-Whitney U test. Kruskal-Wallis test with Dunn’s

multiple comparison test was used with qRT-PCR results. Significant differences in epithelial integrity were analyzed using Fisher´s test. Statistical significance was set to a p-value of <0.05.

3. Results

3.1. Cell attachment and viability on PLCL and PTMC membranes in vitro

The QyQUANT assay illustrated that there was small, yet statistically significant difference on the hUCs attachment on PLCL or PTMC membranes after 1 d of cell implantation (Figure 3A, $p < 0.05$). Live/dead staining confirmed that the majority of cells were viable on both the PLCL and PTMC membranes and that the number of dead cells was negligible after the 1- and 2-week assessment periods (Figure 3B). According to the qualitative analysis, the number of urothelial cells was notably lower on PLCL compared to the PTMC, especially after 1 week of cell culture.

3.2. Expression of urothelium markers

The expression of different urothelial markers was studied after 14 d of cell culture on PLCL and PTMC membranes with qRT-PCR (Figure 4). On both PLCL and PTMC membranes, the hUCs expressed cytokeratins (CK) 7, CK8 and CK19, which are known to be present in all layers of multilayered urothelium. On the PLCL, the expression of CK7 and CK8 was statistically higher compared to the PS and PTMC, respectively ($p< 0.05$). However, the CK19 expression of hUCs was significantly lower on PLCL compared to PS ($p< 0.05$). Additionally, on the PTMC the hUCs expressed all the studied uroplakins, UPIa, UPIb and UPIII, which are more specific markers for urothelial cells. The hUCs on the PLCL expressed only the UPIb marker, whereas, no UPIa and UPIII expression was detected. The expression of UPIa was statistically higher with PS and the expression of UPIb and UPIII was statistically higher with PTMC compared to the PLCL ($p < 0.05$).

3.3. In vivo experiment

During the operation, the PTMC membranes appeared more flexible and were easier to suture and mold into tubular structures around the catheter compared with the PLCL membrane.

The rabbits recovered well after the operation. Most of the rabbits started to eat and drink normally and urinated spontaneously 1–3 days after the surgery. One rabbit from the PLCL group did not urinate and eat normally until four days after the operation. Further, two rabbits from PTMC group died 2 days after the operation, and those rabbits were excluded from the study. Both rabbits underwent autopsy, which revealed no biomaterial related causes of death.

The urethrographic examination performed at 2, 4 and 16 weeks detected no severe strictures, and the radiocontrast agent passed through the defect area in all rabbits (Figure 5). The postoperative spontaneous urination supported our urethrographic findings. The biomaterials in both the PLCL and PTMC groups could not be seen anymore at the 16-week time point.

3.4. Histology

The histological examination showed that the epithelial integrity approached that of the normal urothelial state in both groups (Figures 6 and 7). By week 16, the epithelium was continuous, and, in the majority of samples, the epithelium was stratified (Table 2). Within the PTMC group, the difference in epithelial integrity between the 2- and 16-week follow-up was statistically significant ($p=0.048$). In the sham surgery rabbit group, inflammation was negligible after the 2- and 4-week follow-up. The biomaterials used in this study caused only mild inflammation throughout the follow-up period. The highest scores of inflammatory cells (score 2 [0-3]) were discovered at the 4-week time point in the center defect area in the PTMC group, but the difference between the groups was not statistically significant ($p=0.310$). However, especially in the PLCL group, edema (score 0) and inflammatory cells (score 0) seemed to decrease by the last

16-week time point without a significant difference from the PTMC group (score 1 [0-2] and score 0 [0-2], respectively). No differences in edema or presence of inflammatory cells could be detected in any samples between the groups. Signs of fibrosis varied from mild to moderate during the follow-up. There were no statistically significant differences between the groups. At the 4-week time point, fibrosis was mild in the PLCL group and moderate in the PTMC group (Table 2). In the histological examination, there were no membrane remnants at the 16-week time point in either group.

3.5. Immunohistochemistry

Immunohistochemical staining with the cytokeratin marker (AE1/AE3) demonstrated the formation of *de novo* urothelium (Figures 6 and 7). Hence, in the stainings, no differences between the PLCL and PTMC biomaterial groups were detected at any time points. Further, the *de novo* urothelium developed towards normal stratified urothelium during the assessment period.

4. Discussion

In this study, we investigated the suitability of the PLCL and PTMC membranes for urethral reconstruction in a rabbit model. Reconstruction of severe urethral defects is problematic because additional nonurological tissue grafts are needed, and those operations are highly susceptible to complications. Furthermore, nonurological grafts should fulfill the versatile challenging requirement, from biocompatibility to formable structure.

PLCL was selected because our previous *in vitro* studies have shown its suitability as a growth surface for human urothelial cells.[10,11] It is a biocompatible biomaterial that has been studied in various soft tissue engineering applications with encouraging results.[12,14] Furthermore, Kloskowski *et al*. have previously demonstrated that PLCL was more suitable for ureter segment reconstruction compared with the acellular aortic arch in a rat model.[26] Additionally, PTMC has

been shown to be a biocompatible biomaterial, and it has been studied in particular in soft tissue engineering applications with promising results.[15,19] At least to our knowledge, this is the first study comparing the synthetic biomaterials PLCL and PTMC for urethral reconstruction.

We demonstrated, that the hUCs attachment on PTMC was significantly better compared to PLCL, however, the hUCs remained their viability on both materials, which was expected since both biomaterials are known to be biocompatible. During this study we also evaluated the phenotype of hUCs after two-weeks *in vitro* culturing period on both biomaterials. We analyzed the markers CK7, 8 and 19 since those are generally expressed in multilayered epithelium and throughout all layer in urothelium. Further, the UPs were analyzed due to their specificity for superficial urothelial cells.[21,27] Both biomaterials appeared to support the maintenance of the hUCs phenotype further indicating their potential for urothelial applications. Interestingly, the PTMC seemed to support the expression of uroplakin markers superiorly compared to the PLCL, however, evaluating the significance of these results requires further *in vitro* and *in vivo* studies.

The majority of the rabbits recovered well after membrane implantation and started to eat and urinate within a few days after the operation. One rabbit from the PLCL group had a slight delay in recovering. Two rabbits from the PTMC group died the second postoperative day. These rabbits did not eat after the operation and drank only remotely before they died; however, both rabbits urinated after the operation. The other rabbit had a hematoma at the defect area, but no specific reason could be identified for the death. Nevertheless, we concluded that the deaths were unlikely to be biomaterial-related.

In this study, we also evaluated the applicability of the biomaterial membranes for urological applications. During the operation, PTMC was easier to suture and mold into a tubular structure than PLCL, even though both biomaterials were flexible and easy to handle.

An appropriate biomaterial for urethral reconstruction should not cause disadvantageous tissue effects, such as excessive scar formation, leading to urethral strictures, causing decreased urinary flow and predisposing the patient to urinary tract infections. In our study, the animals were sacrificed 2, 4 or 16 weeks after the operation, and urethrographic examinations were performed to ensure the openness of the urethra. At the 2-week time point, a narrowing of the urethra was detected in both biomaterial groups, but this may be due to the inflexibility of the biomaterial membranes compared to the native urothelial tissue. No severe strictures were detected at the 16-week time point. However, in the PLCL group, a mild narrowing of the urethra was detected at the proximal defect area, whereas the distribution of the radiocontrast agent was uniform when the urethra was reconstructed with the PTMC membrane.

According to a visual inspection after sacrifice, both the PLCL and PTMC membranes were still present after the 4-week follow-up but had fully degraded by 16 weeks, which is consistent with previous degradation studies.[11,17,18] However, at the 2-week and 4-week time points, the PLCL membrane appeared to be more unevenly degraded or more peeled off than the PTMC membranes. PLCL was more rigid than PTMC at 2 and 4 weeks when the defect area was revealed. However, visual inspection showed no substantial differences in the *de novo* urethral membrane after the 16-week assessment period, and there were no macroscopically observable strictures or fibrosis.

The histological results based on the H&E and AE1/AE3 staining showed that the urothelium in both groups developed towards a normal urothelium with regards to integrity and epithelial structure. The positive staining of AE1/AE3 further illustrates the epithelial phenotype of these cells. We hypothesized that the urothelial cells could migrate on the biomaterial membranes from the margin of the graft from the intact urothelium. The development of epithelial integrity was significant in the PTMC group between the 2- and 16-week follow-ups. After 2 weeks, the margin between the defect area and the normal urothelium was evident in both biomaterial groups.

However, the defect margin was no longer distinguishable after 16 weeks, and the *de novo* epithelium showed stratification characteristic of the urothelium.

Invasive treatment always causes fibrosis of some degree. The fibrotic changes on a histological level in our study varied from mild to moderate. The level of observed fibrosis did not cause clinically demonstrable problems, such as urinary retention. Our urethrographic examination proved that the post-operative urethral lumen was open and that the animals started to urinate rapidly after the operation, in other words no severe strictures were detected. In urothelial tissue engineering, the studied biomaterials should not cause severe inflammatory responses.[3] Regarding PTMC, van Leeuwen *et al*. concluded that the tissue reaction on a histological level to PTMC membranes implanted in the mandible was mild and transient.[16] There are no previous data on the tissue response of the urethra to PTMC, but our low inflammatory cell and edema counts suggest that the reaction is mild. Likewise, our results showed mild inflammatory tissue responses in the PLCL group as well. Some results show that PLCL causes an even milder cellular inflammatory response than collagen, which might be related to the slow biodegradation process of PLCL.[28] Based on our histological evaluation, both PLCL and PTMC caused only mild inflammation throughout the follow-up, which establishes their potential.

5. Conclusion

Our aim was to investigate and compare the use of PLCL and PTMC for urethral reconstruction in a rabbit model. In addition, we confirmed the attachment, viability, and phenotype of human urothelial cells on both biomaterials *in vitro*, which further indicated the excellent biocompatibility of the PLCL and PTMC membranes. Our urethrographic examination results and reversion of spontaneous urination after the operation did not reveal clinically remarkable problems, such as strictures. Further, there were no significant differences between the PLCL and PTMC groups in the integrity or structure of the *de novo* urothelium, and therefore, both biomaterials could

be considered potential for urothelial applications. However, PTMC showed significant development of urothelial integrity. Based on our histological evaluation, both PLCL and PTMC caused only mild inflammation throughout the follow-up. Invasive treatment naturally always causes fibrosis to some degree, but the fibrotic changes on the histological level in our study varied only from mild to moderate, and the fibrosis did not cause clinically demonstrable problems. Both biomaterials showed suitability for this purpose without significant differences from each other. In particular, PTMC, which has not been previously investigated for urethral reconstruction and was easier to handle than PLCL, should be considered as a potential biomaterial for urological tissue engineering. The limitation of this research was that we used unseeded biomaterial grafts, and therefore, our next step is to study cell-seeded PLCL and PTMC grafts for urethral reconstruction. Additionally, in the future it would be beneficial to compare these biomaterials to for instance PGA or BAMG, which are one of the most frequently studied biomaterials for urethral tissue engineering with promising results[4,8]. Further, it would be interesting to study different composite biomaterials, as PLCL or PTMC meshes combined for instance with PEG hydrogel[29] for urethral reconstruction in order to facilitate the regeneration of urethra.

6. Acknowledgments

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7. Disclosure Statement

No competing financial interests exist.

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Figures

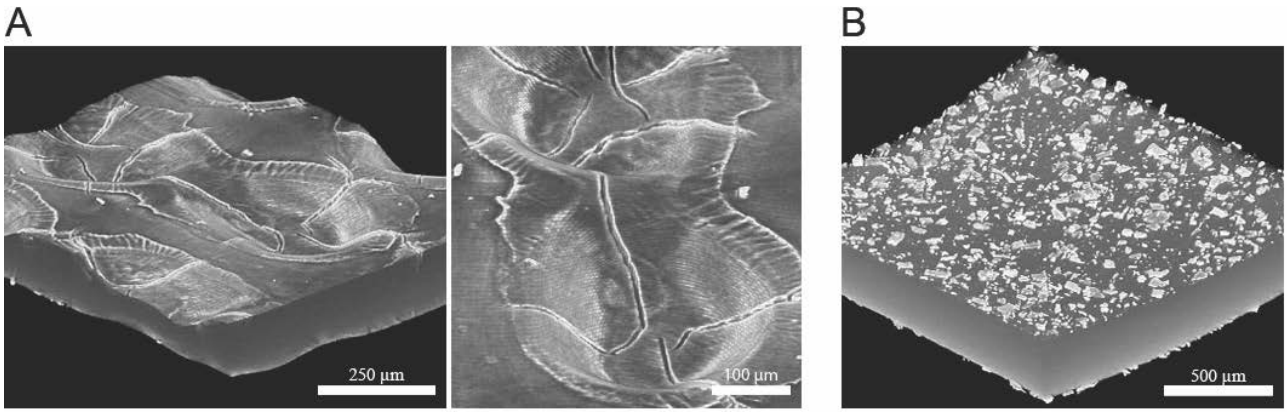

Figure 1: The PLCL (A) and PTMC (B) membranes were imaged with micro CT showing the surface characteristic of the membranes. The maximum diameter of the pit in PLCL membrane is 400 µm. The granules on the PTMC membranes are glucose, which makes the membranes easier to handle.

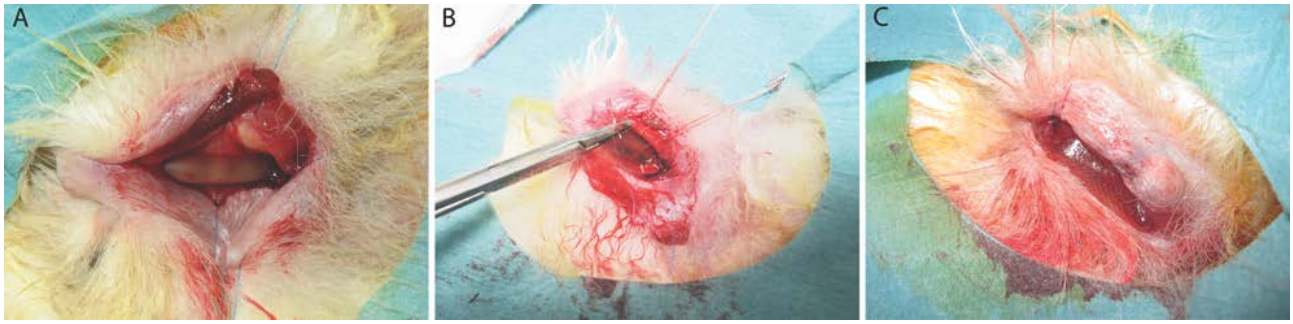

Figure 2: At the beginning of the surgery, the rabbits were catheterized. An incision approximately 2 cm long was made in the inguinal region. The urethra was exposed, and a 2×1-cm oval defect was created in the rabbits' anterior urethra (A). Subsequently, biomaterial membrane of equal size was sutured to the defect area (B), and the biomaterial was aligned with the catheter (C).

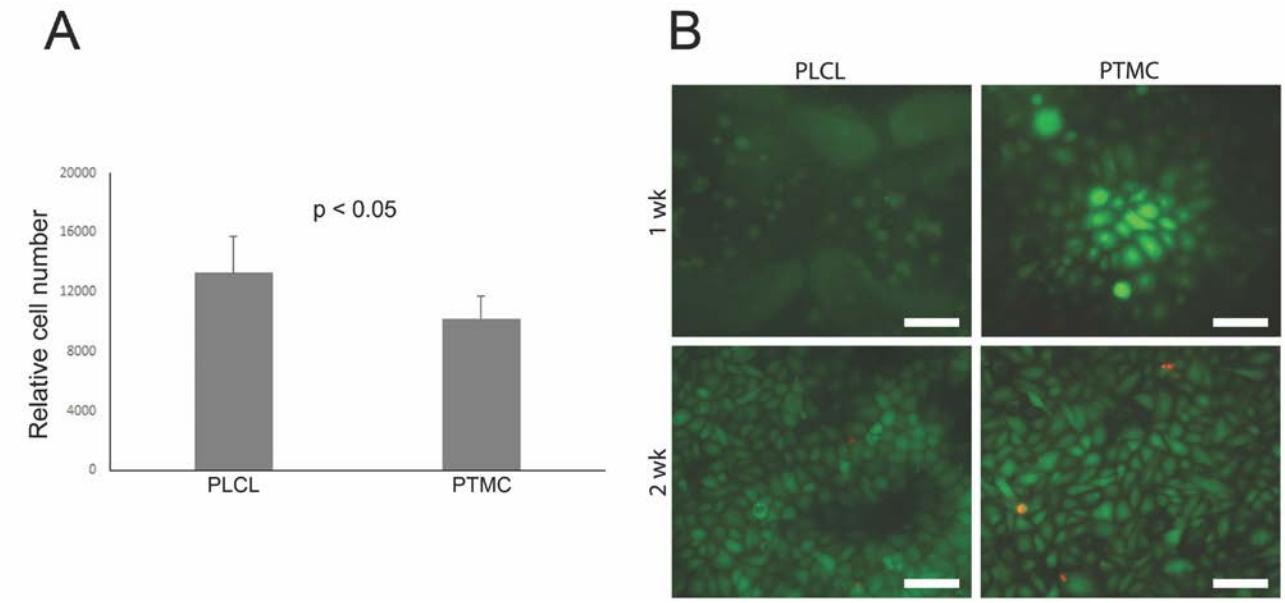

Figure 3: The adhesion of urothelial cells on PLCL and PTMC was assessed after 1 d of cell implantation (A). There was a statistically significant difference on cell attachment between PLCL and PTMC ($p < 0.05$). The figure 3B illustrates representative images of viable (green fluorescence) and dead (red fluorescence) urothelial cells on PLCL and PTMC after 1 wk and 2 wks. Scale bar: 100 μm.

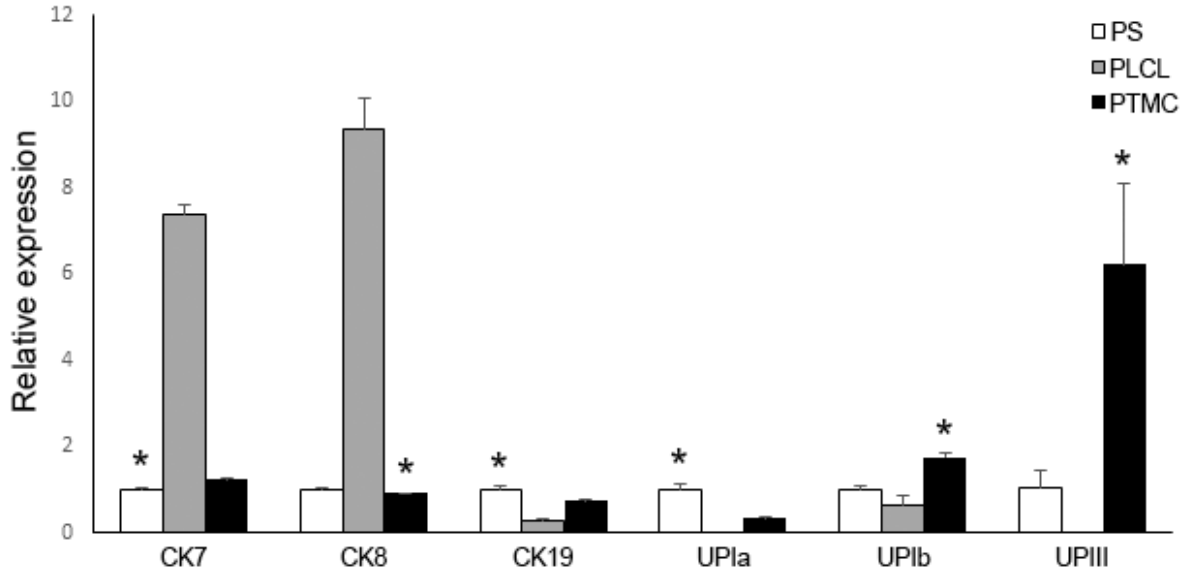

Figure 4: The urothelial cells cultured on PLCL, PTMC and PS expressed different urothelial markers, Cytokeratin (CK) 7, CK8, CK19, Uroplakin (UP) Ia, UPIb and UPIII, after a two-week in vitro assessment period. * $p < 0.05$ with respect to PLCL.

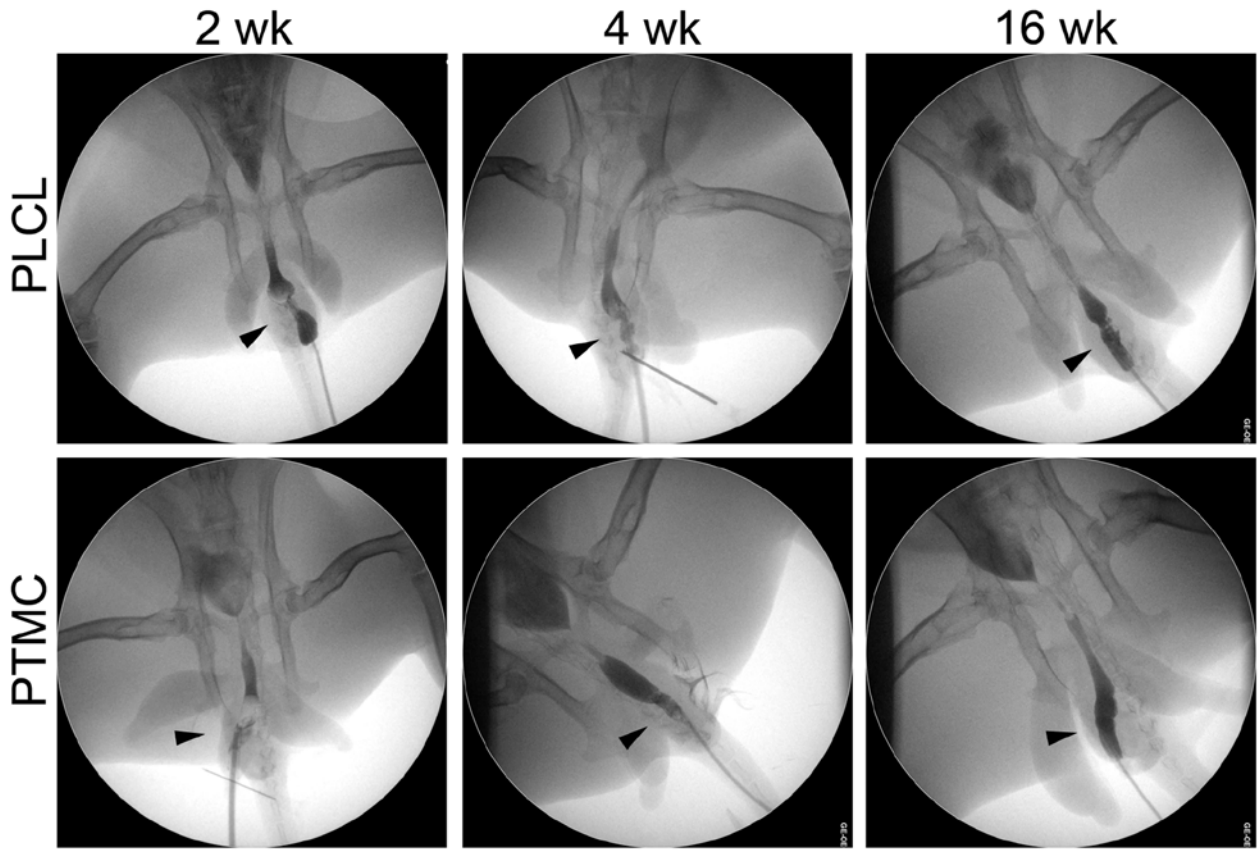

Figure 5: Urethrographic examination illustrated the free passage of the radiocontrast agent through the defect area. After the follow-up, no severe strictures were detected in the PLCL or PTMC biomaterial groups. The arrowhead is indicating the graft area.

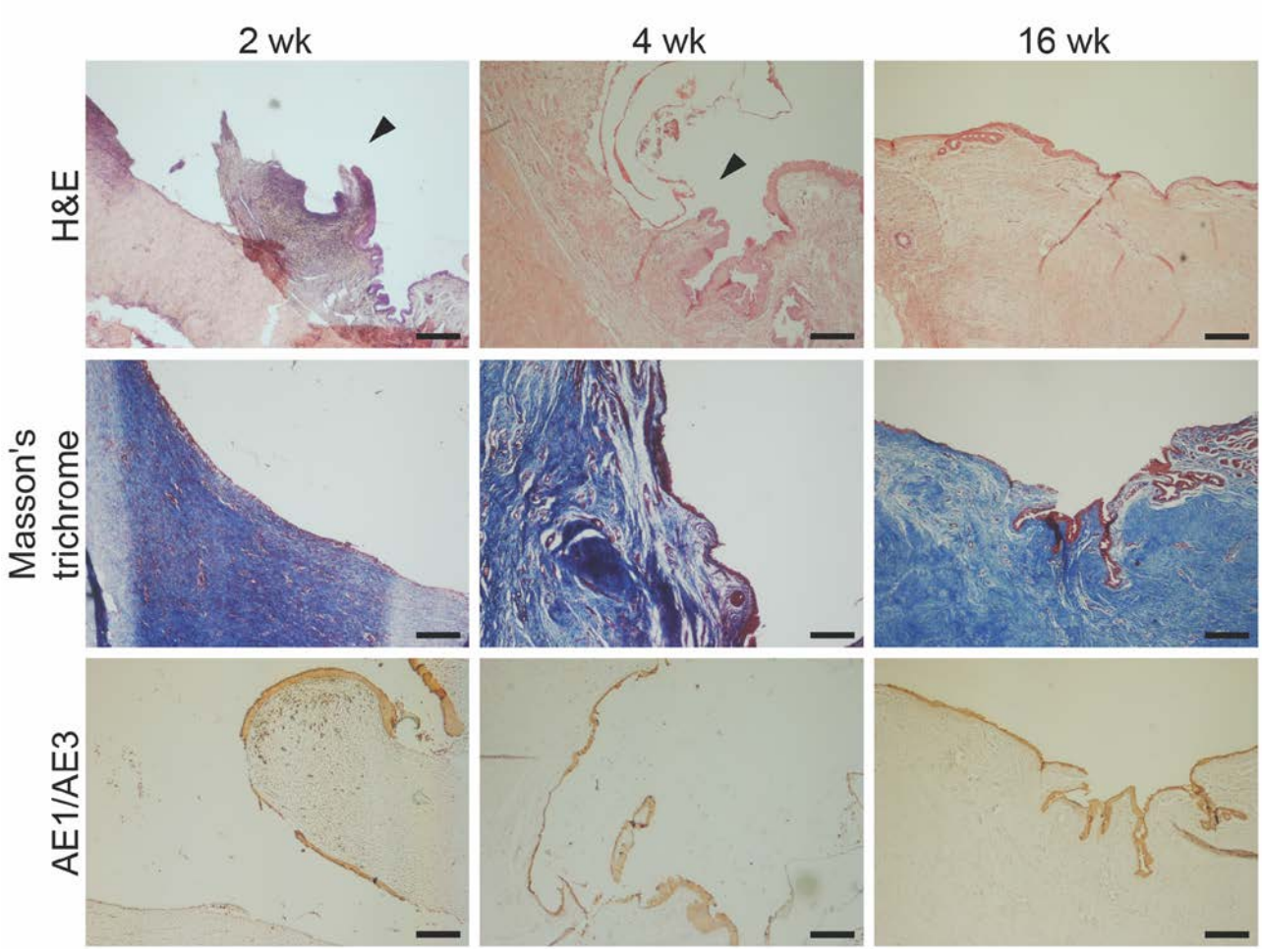

Figure 6: Images show example histological views of the PLCL group at the 2-, 4- and 16-week (n=5) time points stained with hematoxylin and eosin **(**H&E) and Masson's trichrome. The third row of histological images shows pancytokeratin (AE1/AE3)-staining. The implantation area in each panel is located in the centrum of the specimen view. The scalebar is 250 μm and the arrowhead is indicating the margin of natural tissue and biomaterial graft.

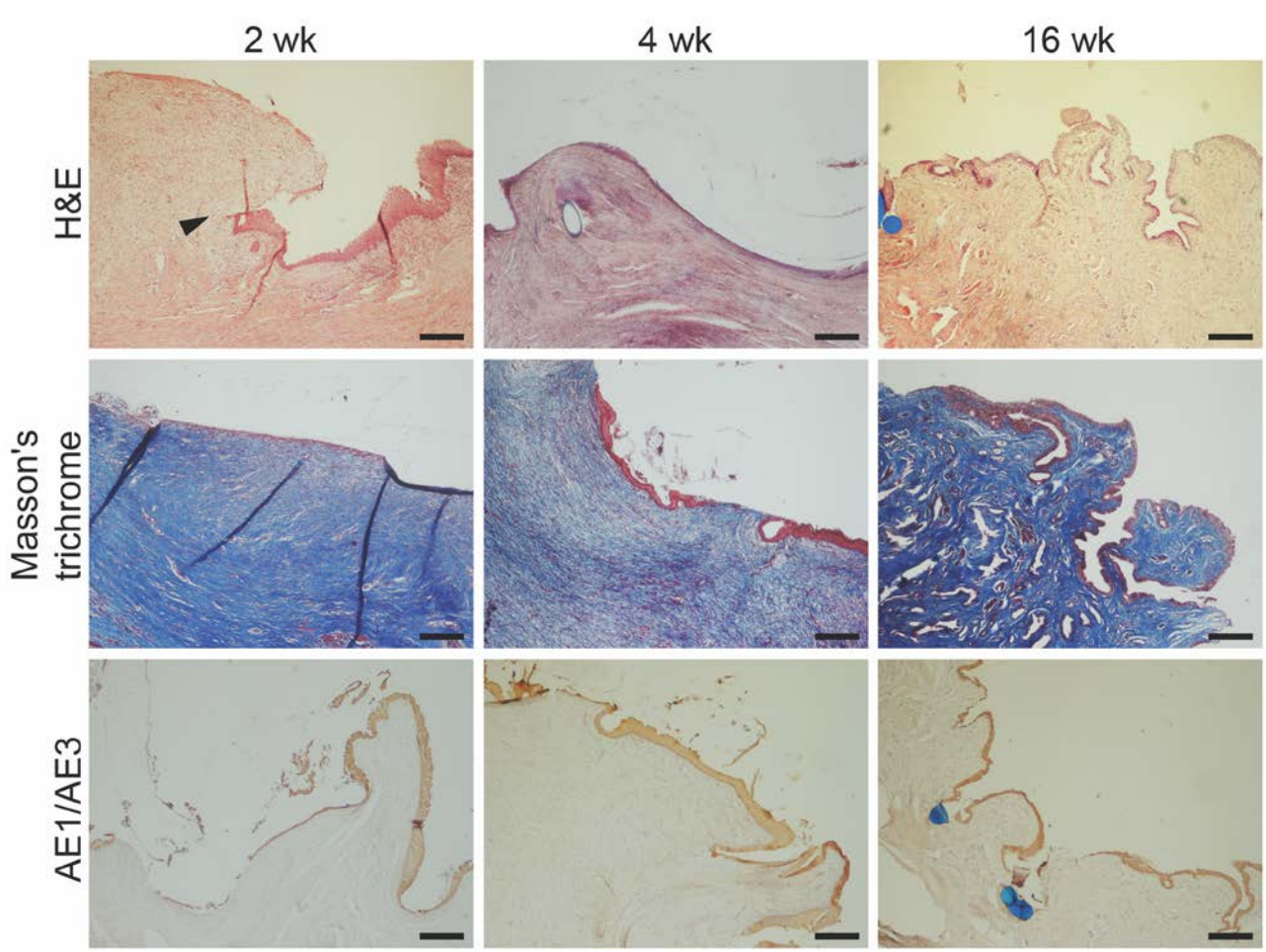

Figure 7: Images show example views of the PTMC group at 2-, 4- and 16-week time points stained with hematoxylin and eosin (H&E), Masson´s trichrome or pancytokeratin (AE1/AE3). The implantation area in each panel is located approximately in the centre of the specimen view. The arrowhead is indicating the margin of urothelium tissue and biomaterial graft and the blue spheres are nonbiodegradable marking sutures. Scalebar 250 μm.

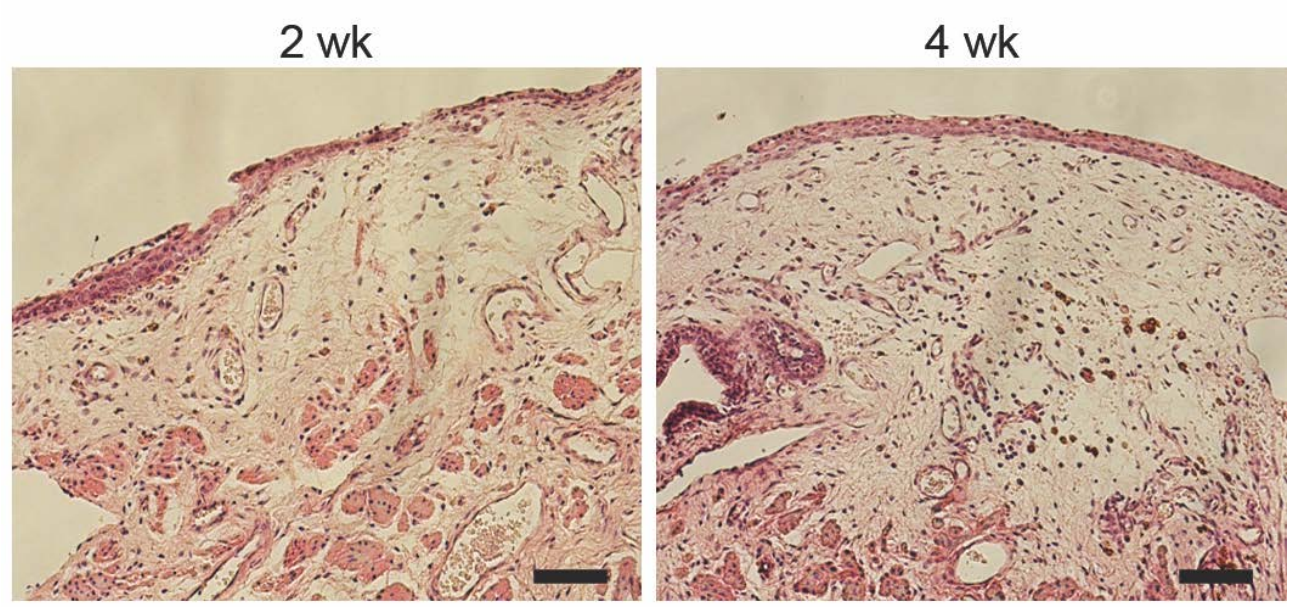

Figure 8: Images show the effect of sham surgery at 2- and 4 week time points. The H&E staining illustrates that the inflammation and fibrosis after 2 and 4 weeks is extremely low indicating the small effect of sham surgery and sutures. Scalebar 250 μm.

Tables

Table 1: The sequences for qRT-PCR primers used in this study.

Name		5'- Sequence -3'	Product size (bp)	Accession number
CK7	Forward	CATCGAGATCGCCACCTACC	80	NM_005556.3
	Reverse	TATTCACGGCTCCCACTCCA		
CK8	Forward	CCATGCCTCCAGCTACAAAAC	68	M34225.1
	Reverse	AGCTGAGGTTTTATTTTGGGACC		
CK19	Forward	ACTACACGACCATCCAGGAC	80	NM_002276.4
	Reverse	GTCGATCTGCAGGACAATCC		
UPIa	Forward	GGGATCTCCAGTTGTGGTGG	80	NM_007000.3
	Reverse	TCTCAGCAAACAGGGACAGG		
UPIb	Forward	AGTCACCAAAACCTGGGACAG	64	NM_006952.3
	Reverse	TGATGGACCATTTACGCCACA		
UPIII	Forward	TCAGTGCAAGACAGCACCAA	65	AB010637.1
	Reverse	GTCCTCCCACCCTCTGTTTG		
RPLP0	Forward	AATCTCCAGGGGCACCATT	70	NM_001002
	Reverse	CGCTGGCTCCCACTTTGT		

Table 2. Epithelial structure and appearance of inflammation or fibrosis after 2, 4 or 16 weeks of follow-up.

	Parameter		Control	PLCL	PTMC	p-value
2 weeks	Inflammation	Cells	1	1 [0-2]	1 [1-2]	0.310
		Edema	1	1 [0-2]	0 [0-1]	0.222
	Epithelial integrity	Discontinous	100 % (1)	60 % (3)	80 % (4)	1.000
		Continous	0 % (0)	40 % (2)	20 % (1)	
	Epithelial structure	None	0 % (0)	20 % (1)	20 % (1)	1.000
		Monolayer	100 % (1)	60 % (3)	60 % (3)	
		Layered	0 % (0)	20 % (1)	20 % (1)	
	Fibrosis		1	1 [1-2]	1 [1-2]	1.000
4 weeks	Inflammation	Cells	0	1 [0-2]	2 [0-3]	0.310
		Edema	0	1 [0-1]	0 [0-1]	0.690
	Epithelial integrity	Discontinous	0 % (0)	60 % (3)	100 % (5)	0.444
		Continous	100 % (1)	40 % (2)	0 % (0)	
	Epithelial structure	None	0 % (0)	20 % (1)	40 % (2)	0.348
		Monolayer	0 % (0)	60 % (3)	60 % (3)	
		Layered	100 % (1)	20 % (1)	0 % (0)	
	Fibrosis (total)		0	1 [1-2]	2 [1-2]	0.067
16 weeks	Inflammation	Cells	-	0 [0-1]	0 [0-2]	0.841
		Edema	-	0 [0-1]	1 [0-2]	0.548
	Epithelial integrity	Discontinous	-	0 % (0)	0 % (0)	1.000
		Continous	-	100 % (5)	100 % (5)	
	Epithelial structure	None	-	0 % (0)	0 % (0)	0.545
		Monolayer	-	40 % (2)	20 % (1)	
		Layered	-	60 % (3)	80 % (4)	
	Fibrosis		-	2 [1-2]	2 [1-2]	0.545