Original Article

Urinary bladder reconstruction using autologous collagenous connective tissue membrane "Biosheet®" induced by in-body tissue architecture: A pilot study

Yasumasa Iimori [a], Ryosuke Iwai [b], Kengo Nagatani [a], Yuka Inoue [a, 1], Marina Funayama-Iwai [a], Mari Okamoto [a], Mio Nakata [a], Keiichiro Mie [a], Hidetaka Nishida [a], Yasuhide Nakayama [c], Hideo Akiyoshi [a, *]

[a] Laboratory of Veterinary Surgery, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58 Rinku-oraikita, Izumisano-shi, Osaka 598-8531, Japan
[b] Research Institute of Technology, Okayama University of Science, 1-1 Ridaicho, Kita-ku, Okayama, 700-0005, Japan
[c] Biotube Co., Ltd., 2-13-11, Shinkawa, Chuo, Tokyo, 104-0033, Japan

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ABSTRACT

Introduction: In-body tissue architecture (iBTA) technology, based on cell-free tissue engineering, can produces collagenous tissues for implantation by subcutaneous embedding a designed mold. The aim of this study was to evaluate the biocompatibility of iBTA-induced "Biosheet®" collagenous sheets, as scaffold materials for bladder reconstruction.
Methods: Canine Biosheet® implants were prepared by embedding molds into subcutaneous pouches in beagles for 8 weeks. A part of canine bladder wall was excised (2 × 2 cm) and repaired by patching the same sized autologous Biosheet®. The Biosheet® implants were harvested 4 weeks (n = 1) and 12 weeks (n = 3) after the implantation and evaluated histologically.
Results: No disruption of the patched Biosheet® implants or urinary leakage into the peritoneal cavity was observed during the entire observation periods. There were no signs of chronic inflammation or Biosheet® rejection. The urine-contacting surface of luminal surface of the Biosheet® was covered with a multicellular layer of urothelium cells 4 weeks after implantation. α-SMA-positive muscle cells were observed at the margin of the Biosheet® implants at 12 weeks after the implantation. In addition, in the center of the Biosheet® implants, the formation of microvessels stained as α-SMA-positive was observed.
Conclusion: Biosheet® implants have biocompatibility as a scaffold for bladder reconstruction, indicating that they may be applicable for full-thickness bladder wall substitution. Further studies are required for definitive evaluation as a scaffold for bladder reconstruction.
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1. Introduction

Reconstruction of the urinary tract using gastrointestinal segments is commonly used in clinical applications in humans [1]. Surgical problems resulting from the use of gastrointestinal tissues can give rise to a wide variety of complications, including metabolic disturbances, urinary tract infections, urine leakage, stone formation, intestinal obstruction and/or malignancy of the intestinal graft [2,3]. It is therefore necessary to develop better scaffolds for urological tissue reconstruction. Synthetic materials have alternatively been used for urinary tract reconstruction [4]. Although synthetic materials such as polytetrafluoroethylene (Gore-Tex) can be

produced with specified properties of strength, and microstructure, these materials lead to chronic inflammatory reactions and promote urinary tract infections and necrosis bladder [5,6].

Collagen-based scaffolds, including bladder acellular matrices (BAM) [7] and porcine small intestinal submucosa (SIS), have been used for bladder reconstruction [8,9]. These have the advantage of maintaining inherent bioactivity, and feature the same tridimensional architecture as the native tissue [10]. Major disadvantages of natural acellular scaffolds are changes to the physiological environment due to decellularization and sterilization processes [11,12], and the possible triggering of an immune response [13,14]. Furthermore, the cost and availability of these materials can be prohibitive, whether they are sourced from animals or cadavers.

In-body tissue architecture (iBTA) is a cell-free tissue engineering technology capable of producing autologous collagenous tissues with any desired shape and appropriate mechanical properties via simple subcutaneous embedding of a specially designed mold [15—17]. It does not involve decellularized procedures, complex *in-vitro* cell management procedures or exceptionally clean laboratory facilities. The sheet-type tissues developed using the iBTA technique are called "Biosheet®." The Biosheet® consists of the fibroblasts and type 1 collagen-rich extracellular matrix [18]. The burst strength was more than 200 mm Hg [19]. Size and thickness of the Biosheet® implants can be changed by altering the molds [20]. In a previous study, autologous Biosheet® implants were successfully implanted as a diaphragmatic repair material in a rabbit model, with suitable mechanical properties and biocompatibility [21]. The scaffolds implanted into the urinary tract are exposed to unfavorable conditions, including the toxic effect of urine [22]. An ideal scaffold for bladder reconstruction would be impermeable to urine, biodegradable, and have biocompatibility [23]. Important biocompatibility factors for scaffolds for bladder reconstruction include the absence of a foreign body reaction from the bladder, the absence of excess inflammation, and the capability for the material to become an appropriate scaffold for cell migration [24].

In urology, dogs are one of the most frequently used large animals in experiments with implantation of tissue-engineering scaffolds [25]. Furthermore, research in dogs can contribute to human medical sciences and also to veterinary medicine [26]. The aim of this pilot study was to evaluate the biocompatibility of Biosheet® implants as a scaffold for bladder reconstruction in a dog model.

2. Materials and methods

2.1. Ethical approval

All research protocols were approved by the ethics committee of Osaka Prefecture University with animal experience (No. 29—81, 29—169). We performed the experiments in accordance with the guidelines established by the National Institutes of Health for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Animal model

Four healthy adult beagles were selected for this study (1—2 years old, female, 9.3—9.9 kg). The dogs were obtained from Oriental Yeast Co., and were housed individually in stainless-steel cages in a controlled environment with free access to water and food until 12 h before anesthesia. Supplementary file summarizes the animal procedure schedule.

2.3. Preparation and implantation of Biosheet® implants

Biosheet® implants were prepared according to the method published previously [20]. The porous cylindrical molds for preparation of the Biosheet® implants were assembled by inserting multiple stainless-steel slits (outer diameter 1 mm) into an acrylic tube, and caps (5 cm long, 2 cm outer diameter, 1.8 cm inner diameter) (Fig. 1a). Autologous Biosheet® implants were prepared by embedding specially designed molds into subcutaneous pouches in the dogs (n = 4, four molds per dog). The dogs were injected subcutaneously with atropine sulfate (0.025 mg/kg, Fuso Pharmaceutical Industries, Osaka, Japan) prior to anesthesia. Anesthesia was induced by intravenous administration of propofol (6 mg/kg, Intervet, Osaka, Japan) following intravenous injection of midazolam (0.2 mg/kg, Dormicum, Astellas Pharma, Tokyo, Japan) and butorphanol (0.2 mg/kg, Vetorphale, Meiji Seika Pharma, Tokyo, Japan). An endotracheal tube was placed into the trachea to facilitate control of respiration. Anesthesia was maintained with 1.5—2.0% isoflurane (Mylan Seiyaku, Tokyo, Japan) and oxygen. Cefazolin (Cefamezin α, LTL Pharma, Tokyo, Japan) was administered intravenously prior to the operation. The molds were extracted 8 weeks after the implantation, and the collagenous tubes formed on the acrylic tubes were gathered by removing the molds and trimming the surrounding peripheral tissues (Fig. 1b). Rectangular-shaped Biosheet® implants (3 × 5 cm long, ca. 1 mm thick) were obtained by cutting the tubular tissues longitudinally (Fig. 1c). The Biosheet® implants harvested from the subcutaneous portion were white, thin, flexible sheets and mainly consist of the fibroblasts and the collagen as described in previous reports [18,21]. The Biosheet® implants were immediately soaked in 70% ethanol for 10 min and washed with physiological saline prior to bladder implantation.

The urinary bladder was emptied before surgery using a urethral catheter, with an approach through a 5 cm caudal midline abdominal skin incision. A piece of the ventral wall of the bladder body having full thickness was excised (2 × 2 cm) (Fig. 1d). A same sized piece autologous Biosheet® (Fig. 1e) was sutured to the edge of the native bladder using a simple continuous suture pattern of 4-0 polydioxanone material (Monoplus; B. Braun Aesculap Japan, Tokyo, Japan) (Fig. 1f). The urethral catheter was maintained for 4 h postoperative and was then removed. Removing of the molds and implantation of the Biosheet® implants to the bladder were on the same day. The Biosheet® implants were extracted under anesthesia at 4 weeks (n = 1) and 12 weeks (n = 3) after the implantation. After extraction of the Biosheet® implants, the urinary bladders were sutured using a simple continuous suture pattern of 4-0 polydioxanone material. All the dogs were rehabilitated and rehoused after this study.

2.4. Ultrasonography and urography

Ultrasonographic examination was performed at the following time points: ≤ 1 day prior to the implantation (i.e., baseline), 1, 3 days, and 1, 2, 3, 4, 8 and 12 weeks after the implantation. An Aplio XG scanner (Toshiba Medical Systems, Tokyo, Japan) with a convex multifrequency abdominal probe was used to perform ultrasonography examination. Ultrasonography was performed to check for urine leakage, calculi, hematoma, hypertrophy and calcification in the reconstructed bladder. Urography was performed postoperatively at 4 and 12 weeks after the implantation. Urography was made at 0, 1, 5, 10, 15, and 20 min after injection of contrast medium, to check for urine leakage and urine storage. The dogs were given iohexol (Omnipaque; Daiichi Sankyo, Tokyo, Japan) at
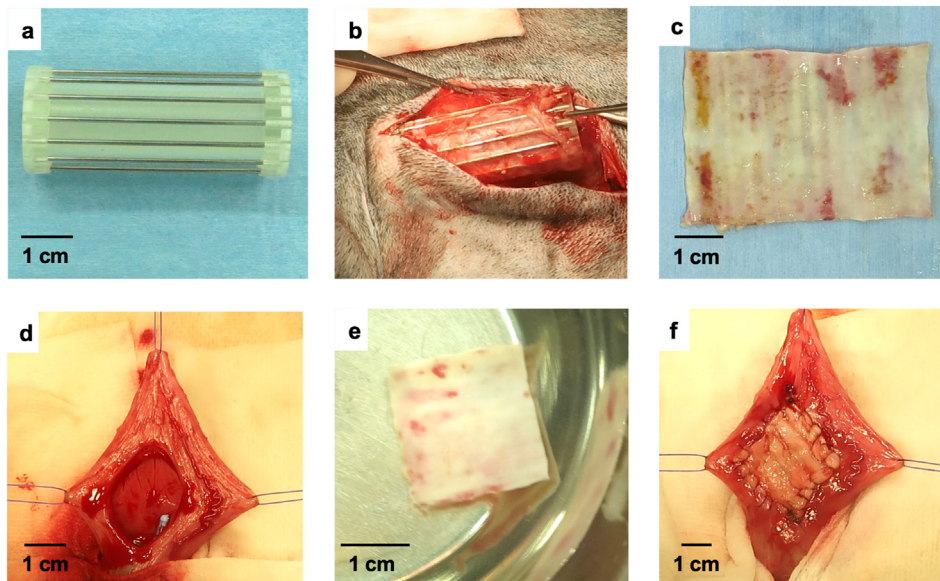
Fig. 1. Photographs of the preparation and implantation of Biosheet® implants. (a) Mold for Biosheet® preparation (b) Mold encapsulated completely with Biosheet® tissue 8 weeks after implantation. (c) Biosheet® covered the mold after trimming to remove fragile and redundant tissue. (d) Urinary bladder wall after dissection (2 × 2 cm) (e) Trephined Biosheet® (2 cm in diameter) (f) Urinary bladder after implantation of Biosheet®.

doses of 600 mg of iodine/1 kg of body weight by rapid IV injection; the urinary bladders of the dogs were empty prior to these injections.

2.5. Histological examination

The extracted specimens were fixed with 10% formalin, embedded in paraffin, and sliced into longitudinal sections of thickness 3–5 μm. Sections were stained with routine hematoxylin and eosin (H&E), and Masson's trichrome (MT) stain for collagen assessment.

The deparaffinized sections were microwaved for 12 min at 360 W in 0.1 M citrate buffer (pH 6.0) for paraffin wax removal and antigen retrieval. Subsequently, the sections were washed twice for 10 min in diluted water (DW) and blocked in 1.0% bovine serum albumin in PBS at room temperature for 1 h, then incubated with mouse monoclonal anti α-SMA (1:200; Abcam) antibody overnight at 4 °C. The antibody cross-reacts with α-SMA in dog [27]. After washing twice for 10 min in DW, sections were incubated with Alexa fluor 594 rabbit anti-mouse IgG antibody (1:1000; Life Technologies) at room temperature for 2 h, followed by DAPI (Life Technologies) for 5 min, in order to stain the nuclei. Slides were then analyzed by fluorescent microscopy.

3. Results

3.1. Imaging

Urography and ultrasonography did not show any urine leakage or bulging of Biosheet® implants during the observation period (Fig. 2). There were no calculi, and no hematoma, calcification, or metaplasia in the urinary bladder according to ultrasonography during any observation period (Fig. 2).

3.2. Macroscopic observation

Macroscopically, there were no urinary leaks or extravasation of urine into the abdominal cavity. The bladders were uniformly dilated upon distention. The surfaces of the Biosheet® implants were completely covered with thin, newly formed tissue and visible angiogenesis, even at 4 weeks after implantation (Fig. 3a and b). Borders of the Biosheet® implants could not be clearly distinguished visually from the urinary bladder outer surface at 12 weeks after implantation (Fig. 3c and d). There were no calculi or necrosis of the Biosheet® implants at 4 and 12 weeks after implantation.

3.3. Histological findings

At 4 weeks after implantation, a multi-cell layer of regenerated transitional epithelium completely covered the Biosheet® forming the mucosa of the substituted portion (Fig. 4). The connective tissue was visible under the epithelium, with neovascularization (Fig. 4 b). Small foci of lymphoid and macrophage infiltration were also seen. The Biosheet® site was infiltrated with a few inflammatory cells, and adequate neovascularization occurred.

Although most Biosheet® implants were degraded and indistinguishable from the native bladder, in the center of the implanted site the Biosheet® implants retained their identity deeper in the submucosa at 12 weeks after implantation (Fig. 5). The tissues on the Biosheet® implants composed confluent, well-differentiated, urothelial layers at 12 weeks after implantation. The organization of the submucosa was better than at 4 weeks after implantation (Fig. 5b and c). There were few inflammatory cells in the Biosheet® implants at 12 weeks after implantation. Spindle shaped cells with eosinophilic cytoplasm had infiltrated inwards at the borders, originating from native muscle tissues (Fig. 5d and f). Immuno-histological analysis revealed that these spindle cells were α-SMA positive (Fig. 5g and i). Microvessels which stained α-SMA-positive were observed at the center of the Biosheet® implants (Fig. 5e and h). There were no necrosis or calcification of the Biosheet® implants at 12 weeks after implantation. There was no sign of Biosheet® rejection from surrounding the bladder tissues during any observation period.

4. Discussion

We have demonstrated in this pilot study the implantation of autologous Biosheet® implants having biocompatibility as a
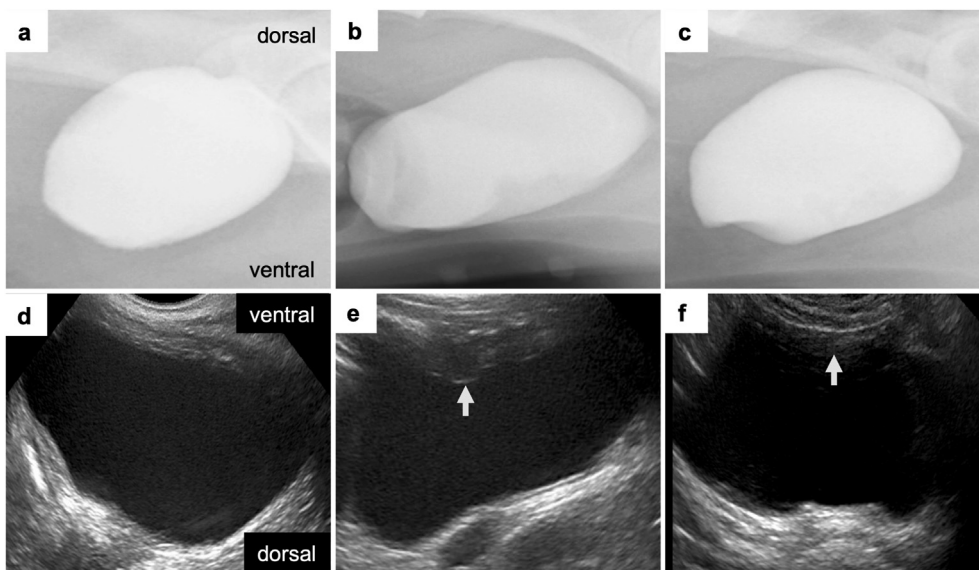
Fig. 2. Typical images of urography and ultrasonography after implantation of Biosheet® implants. (a, d) Images of the urinary bladder at 0 days after implantation. (b, e) Images of the urinary bladder at 4 weeks after implantation. (c, f) Images of the urinary bladder at 12 weeks after implantation. White arrows indicate the implantation site of the Biosheet® implants.
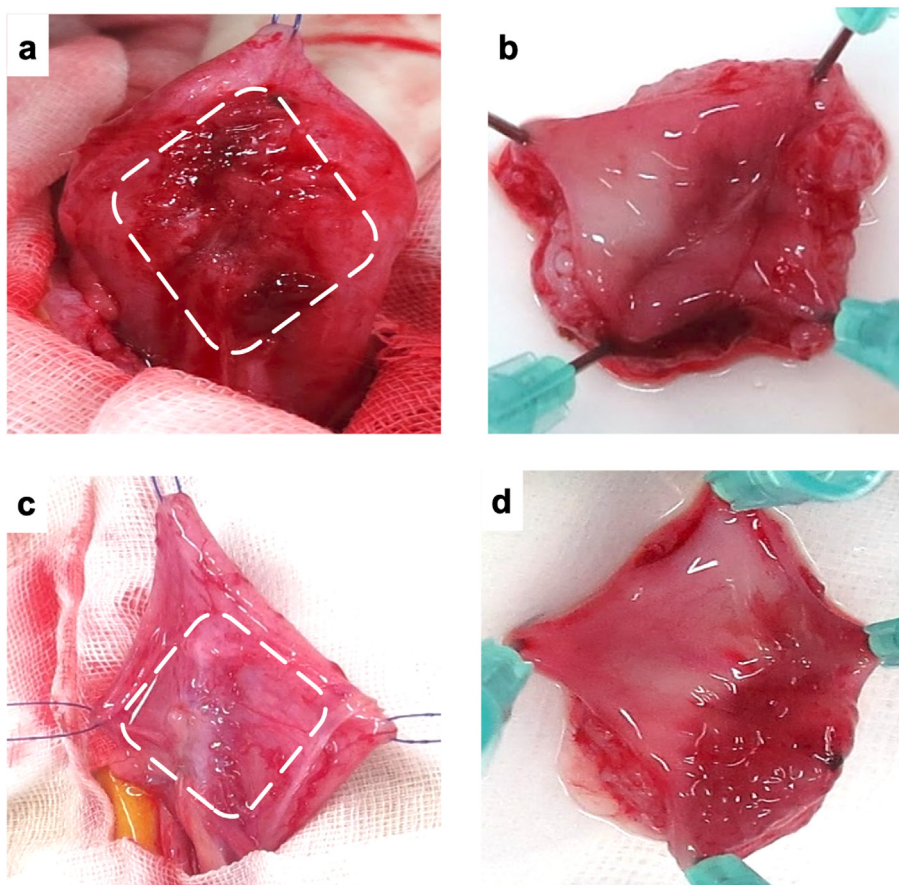

Fig. 3. Macroscopic view of Biosheet® implants in the urinary bladder. (a, c) Urinary bladder at 4 and 12 weeks after implantation. (b, d) Mucous membrane of the Biosheet® implants at 4 and 12 weeks after implantation. The broken line indicates the implantation site of the Biosheet® implants.
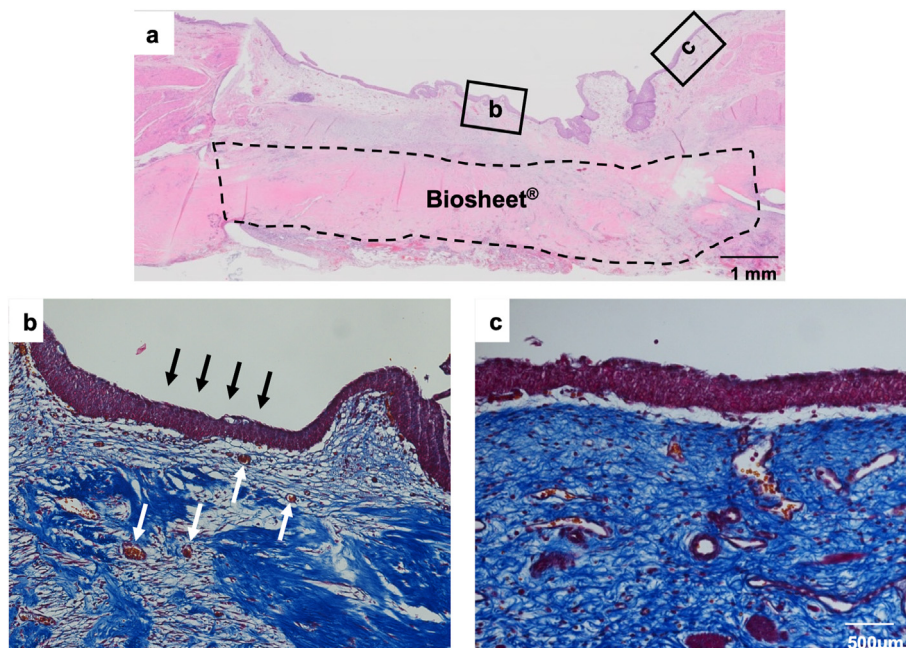
Fig. 4. Histology of Biosheet® at 4 weeks after implantation. (a) Hematoxylin—eosin staining. Complete view of the Biosheet®. (b, c) Masson's Trichrome staining. Collagen is stained blue; nuclei are stained black. Mucosa and submucosa of the Biosheet® and native bladder are shown. Stratified urothelium covered the entire graft surface of the Biosheet® (black arrows). Neovascularization was observed in the loose connective tissue on the luminal (white arrows).

scaffold for bladder reconstruction in a dog model. Four weeks after implantation, the Biosheet® was covered with multi-layered urothelium. Previous reports found that epithelialization of the SIS and BAM graft surface was complete by 3—4 weeks, with normal transitional histology [28,29]. Migration of urothelium to the Biosheet® is similar to previous studies [28,29] that had implanted natural collagen-based scaffolds in the bladder. Biosheet® implants are capable of acting as an effective scaffold for the migration of urothelial cells.

Autologous Biosheet® implants do not elicit detrimental chronic inflammation, or any signs of rejection. Implantation of synthetic material resulted in severe inflammatory and foreign body reactions within the implant and also in the surrounding interface between the implant and host tissue, at 12 weeks after the implantation [30]. A previous report found that dystrophic calcifications of SIS grafts were associated with a largely chronic inflammatory reaction, and occasional foreign body giant cell reaction [29]. The biodegradable materials including polyglycolic acid, polylactic acid, and copoly (lactic/glycolic) acid are attractive bioengineering approaches for bladder reconstruction [31]. However, they can induce foreign body reaction, and calculi formation when they are implanted in the bladder [32]. Biosheet® implants did not cause any detectable complications such as foreign body reactions, necrosis or calcification of the bladder. This is clearly because any inflammatory response to the Biosheet® implants was minimal. Previous reports indicated allogeneic Biosheet® implants were well tolerated in cornea, trachea, and abdominal wall with a little inflammatory response [18,33,34]. Allogeneic Biosheet® implants could be applicable to bladder reconstruction.

Beneath the epithelium, neovascularization occurred in the connective tissue and Biosheet® at 4 weeks after implantation. Immunohistological analysis found α-SMA positive cells with eosinophilic cytoplasm ingrowth from the borders inwards, originating from the native detrusor muscle at 12 weeks after implantation. Smooth muscle cells are rich in myofilaments; their cytoplasm stains eosinophilic [35]. The α-SMA positive cells

observed in the Biosheet® implants may be smooth muscle cells. A previous report found adequate angiogenesis and migration of urothelium resulting in proliferation and migration of smooth muscle cells [11]. These cellular events indicate the remodeling process of the bladder.

Biosheet® implants were remodeled by urothelium, and smooth muscle cells. However, we could not prove the Biosheet® implants replaced by the surrounding bladder tissue in the present study. There were no mature muscular tissues in the Biosheet® implants at 12 weeks after implantation. Nerve regeneration is also important for functional bladder replacement. The signals that control the detrusor muscle and urethral sphincter are conducted in the bladder tissue through nerve fibers [36]. Further long-term studies are needed to assess whether Biosheet® implants can regenerate the function of the bladder.

The Biosheet® implants remained evident in the submucosa at 12 weeks after implantation. The rate of resorption of the SIS is rapid, with as much as 90% of the scaffold replaced by native bladder tissue within 4 weeks [37]. The rapid degradation of the graft led to smooth muscle regeneration [23]. The SIS elicits an immunological reaction [38], resulting in inflammation and rapid degradation. We suggest that biodegradation of the Biosheet® implants are slow due to the autologous implantation.

Strategies exist for altering the Biosheet® implants to allow for more rapid degradation. First, there is the possibility of implanting xenogeneic Biosheet® implants. A previous report found that bovine Biosheet® implants treated with 70% ethanol were accepted into the abdominal wall in dogs [39]. Xenogeneic Biosheet® implants elicit an immunological reaction [39], resulting in inflammation and rapid degradation in bladder reconstruction. Second, the thickness of the Biosheet® implants can be controlled by changing the size of the gap in the molds [20]. Thinner or smaller material results in faster degradation and less encapsulation [40]. Thinner Biosheet® implants can be expected to degrade more rapidly. Third, the properties of Biosheet® implants change in response to alcohol concentration [41]. We treated Biosheet®
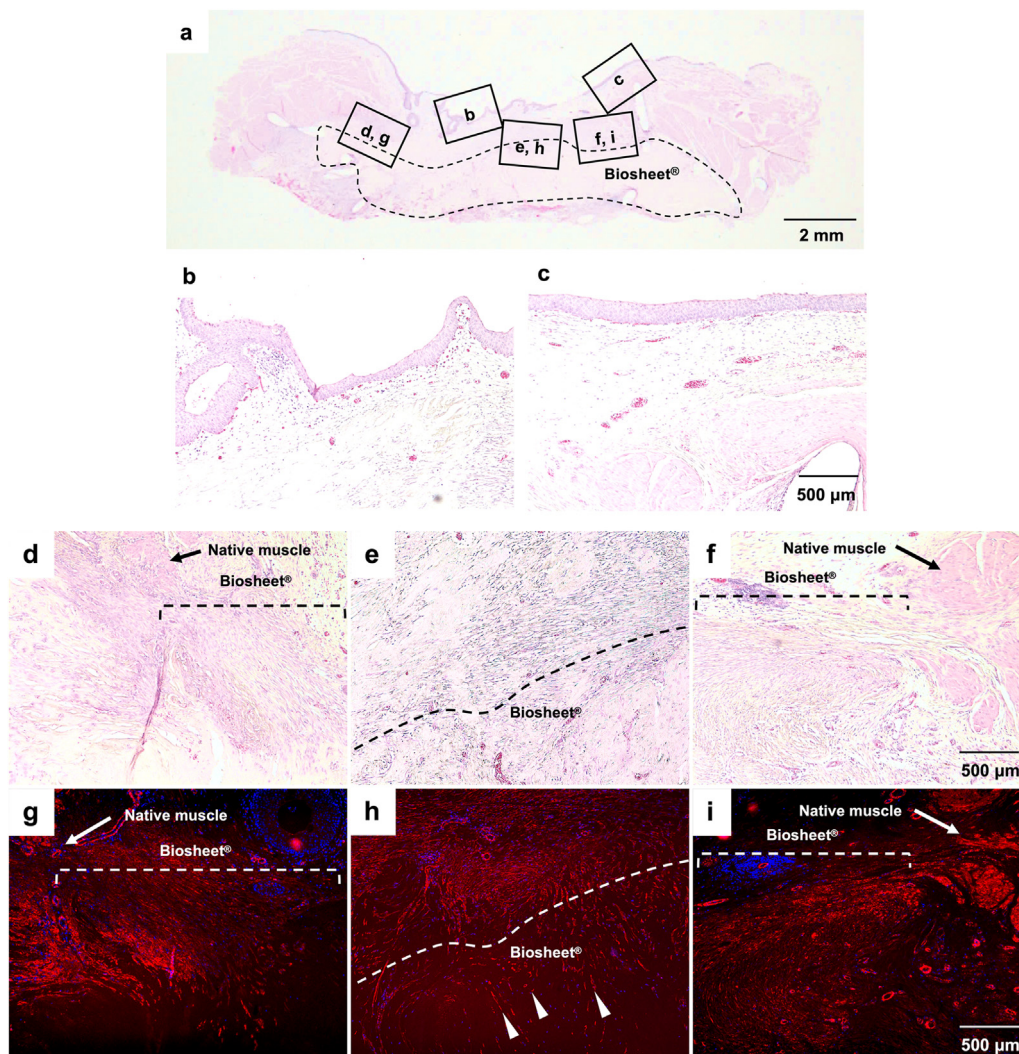
Fig. 5. Histology of Biosheet® implants at 12 weeks after implantation. (a) Complete view of the Biosheet®. (b, c) Mucosa and submucosa of the Biosheet® and native bladder (d, f) Margin of the Biosheet® and bladder. (e) Center of the Biosheet®. (g–i) Immunohistochemical staining for α-smooth muscle actin (α-SMA). Red: α-SMA, Blue: DAPI staining for cell nuclei. (g, i) Cells which stained positive for α-SMA recruited from native muscle tissues to the Biosheet®. (h) Microvessels that stained α-SMA-positive were observed at the center of the Biosheet® (white arrow heads).

implants with 70% ethanol solution before implantation to increase their strength. Faster biodegradation is a goal of future research into Biosheet® implants for bladder reconstruction.

Several limitations of this study are apparent. First, this was not a comparative investigation. There was no control group in this study. Further studies are needed to compare other biomaterials such as decellularized extracellular matrices and biodegradable materials. Second, there was no mechanical resistance testing before implantation of the sheet or at extraction because of the small size of the Biosheet® implants. Third, we did not perform functional testing. Forth, the histological analysis was performed using only one dog at 4 weeks after the implantation. Additional studies, including assessments of large sized patches, and mechanical and functional evaluations, would be required to fully evaluate necessary to determine definitively the potential of Biosheet® implants as scaffold materials for bladder reconstruction.

5. Conclusion

Autologous Biosheet® implants fabricated using in-body tissue architecture technology exhibit biocompatibility as scaffold materials for bladder reconstruction in a dog model. Further experimental studies are required for full evaluation of Biosheet® implants as scaffold materials for bladder reconstruction.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2020.10.006.

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