Bladder Tissue Engineering Using Biocompatible Nanofibrous Electrospun Constructs

Feasibility and Safety Investigation

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Purpose: To investigate the feasibility and safety of using biocompatible, nanofibrous electrospun polycaprolactone (PCL) and combination of polylactic acid (PLLA) and PCL mats in a canine model.

Materials and Methods: Plasma-treated electrospun unseeded mats were implanted in three dogs. The first dog was sacrificed after 3 months and the second and third ones after 4 months, and then, the graft was examined macroscopically with subsequent morphological and histochemical evaluation.

Results: Both films showed high levels of cell infiltration and tissue formation, but body response to PLLA/PCL mat in comparison to PCL mat was very low. All three implantation models showed the same light microscopic morphology, immunohistochemistry, and scanning electron microscopy results; nevertheless, only the PCL/PLLA model showed favorable clinical results.

Conclusion: Based on these data, nanofibrous PLLA/PCL scaffolding could be a suitable material for the bladder tissue engineering; however, it deserves further investigations.

Keywords: urinary bladder, tissue engineering, biocompatible materials, polymers

INTRODUCTION

Bladder is a unique organ in possessing a specific structure. The bladder wall includes three layers, namely mucosa, submocusa, and detrusor muscle. Detrusor consists of smooth muscle cells that have capability of urine storage in filling phase and contraction in voiding period. This complex configuration and communication with sympathic, parasympathic, and somatic nerves make the bladder a challenging organ to be substituted.

The bladder is susceptible to a variety of possible injuries and anomalies. In addition to congenital abnormalities, other tissue damages or losses, such as cancer, trauma, infection, and inflammation, highlight the need for tissue reconstruction.

Bladder reconstruction is done using a segment of the gastrointestinal tract as an alternative matrix for the bladder augmentation or replacement.[1] Reconstruction of the bladder with autologous non-urologic tissues does not provide the entire function. Furthermore, there are some important complications that limit the use of these natural matrices, such as metabolic abnormalities, perforation, malignancy, and infection.[2]

To overcome the problems related to the using of the intestinal segments for the urinary tract reconstruction, many efforts have been made using several biological and synthetic materials, such as the de-epithelialized intestinal segments, seromuscular intestinal segments, dura mater, peritoneum, and fascia, which have resulted in varying degrees of tissue reconstruction.[3] The use of naturally-derived agents, including lyophilized dura and submocusa of porcine small intestine and placenta could result in contraction for an unknown period of time.[4]

Recently, attention has been turned toward auto-augmentation and ureterocystoplasty. Auto-augmentation has had disappointing outcomes in long-term follow-up and ureterocystoplasty requires severely dilated ureters. De-epithelialization of the bowel segment may lead to growth of mucosal layer; however, shrinkage of graft and re-epithelialization of afore-mentioned bowel segment are the main limitations.[5] Insufficient proper native bladder tissue and the distrust in the existence of healthy tissue in the involved bladder resulted in major difficulties in the urinary tract reconstruction using native tissue.

Previously, biological synthetic materials, such as gelatin sponge, polyvinyl, and polytetrafluoroethylene (Teflon) have been used for the bladder reconstruction, but they had no favorable outcomes. The main reason for their failure was body reaction to the foreign agents.[6,7] Permanent synthetic material was concomitant with calcification and mechanical problems.

Recently, some investigators have focused their attention on tissue engineering and using biocompatible synthetic materials for soft tissue substitution. There are two forms of the bladder tissue engineering. The first one is cell-based, in which the isolated and cultured primary cells are seeded on natural or synthetic scaffold and then transplanted into the host, and subsequently the graft regeneration and maturation could be continued in vivo. The second form is cell independent and the acellular matrix or biodegradable, biocompatible scaffolding is transplanted into the host. This acellular matrix is used as a mechanical support for native cells to infiltrate and initiate tissue regeneration. Acellular collagen matrix has many growth factors expected to promote tissue regeneration,[8] but according to literature, this matrix, seeded or unseeded, has not improved tissue regeneration.[9,10] Atala and colleagues produced an effective bladder tissue by using cell implanta-

tion on collagen/polyglycolic acid scaffold. They achieved notable results in compliance, leak point pressure, cellular structure, and phenotypical characteristics in some later cellular transplantation on synthetic scaffold.[11]

Synthetic polymers due to their reproducibility in synthesis, also having appropriate mechanical properties, are the best candidates for matrix synthesis. Furthermore, it is possible to process the polymers to achieve desirable configuration. The application of synthetic/polyglactin composite may lead to chronic infection, foreign body reaction, implant shrinkage, and rejection of the implant.[12,13] Therefore, it is important to investigate the body response to the implanted synthetic unseeded scaffold to check the suitability of using synthetic scaffold for the regeneration of the tissue of interest. Previously, Rohman and associates reported that cells may show preferential growth on materials displaying mechanical properties that most closely represent those of the derived tissue.[14]

In this study, we investigated the feasibility and safety of using nanofibrous electrospun polycaprolactone (PCL) and combination of polylactic acid (PLLA) and PCL mat as a supportive substrate. We also evaluated the graft morphologically and immunohistochemically after implantation in dogs.

MATERIALS AND METHODS

Three adult intact female mongrel dogs weighing between 20 and 25 kg and aged 2 to 3 years were used in this study. The dogs were determined to be healthy based on the results of physical examination and complete blood count. Animals were treated for possible internal parasites (Caniverm®, Bioveta, A. S. Czech Republic, 1 tablet/10 kg per os). They were kept under indoor-outdoor system during the experiment period. They were fed by a balanced commercial dry maintenance diet (Friskies, Purina, Marne-la-Vallee, France) once a day, and water was offered ad libitum. The experimental protocol was approved by the Institutional Animal Care and the Use Committee of Urology and Nephrology Research Center (UNRC) in Iran.

The dogs were premedicated with xylazine-HCl (xylazine 2%, Alfasan International BV, Woerden, the Netherlands) (2 mg/kg) intramuscularly. This was followed by induction of general anesthesia with intravenous injection of the ketamin-HCl mixture (ketamin10%, Alfasan International BV) (10 mg/kg) and diazepam (ZEPADIC®, Caspian Tamin Pharmaceutical Co., Rasht, Iran) (0.5 mg/kg), and maintained by the same drugs.

The urinary bladder was approached through a caudal midline abdominal incision, and linear cystotomies of the bladder dome were done. Augmentation cystoplasty was performed with the scaffolds. After preparation of the PCL and PCL/PLLA nanofibrous scaffold, PCL scaffolds were transplanted to the bladder of the first and second dogs and PCL/PLLA scaffold was transplanted to the third dog. A single layer of continuous interlocking sutures with 4-0 polyglycolic acid was used for the anastomosis. The omentum was then placed over the graft and secured to the bladder with a simple interrupted suture of the 4-0 polyglycolic acid material before the abdominal incisions were routinely closed in three layers (Figure 1). All dogs received teramadol for 3 days and an antibiotic for 14 days. A urethral Foley catheter was placed in the bladder for 10 days to void the urine leakage and relieve tension on the suture line.

All the animals underwent abdominal ultrastenography to evaluate urine leakage on the 3rd

and 7th postoperative days, then monthly afterwards. Complete blood count, blood urea nitrogen, and serum level of creatinine were tested twice a week for two weeks, and then monthly after the operation.

The first dog was sacrificed after 3 months and the second and third ones 4 months later. The graft was investigated macroscopically and then evaluated for light and electronic microscopic morphology as well as immunohistochemistry characteristics.

Fabrication and Preparation of PCLL and PLLA Nanofiber Scaffold

Poly ε-caprolactone/collagen nanofibrous hybrid scaffolds were prepared by double jet electrospinning method, as described by Khademhosseini and colleagues and Matthews and associates.[15,16] Briefly, PCL was obtained from Sigma-Aldrich (Milwaukee, WI, 1 mol, USA). The average molecular weight of PCL was 80 000 g. Chloroform and N, N-dimethylformamide (DMF) were purchased from Sigma (St. Louis, MO, USA). These materials were used as received without any further purification. Poly caprolactone was dissolved in chloroform at room temperature and DMF was added to the chloroform just before the electrospinning process. Many studies have shown that DMF helps during the electrospinning and the formation of electrospun fibers.[17,18] Experiments were conducted at chloroform to DMF ratio of between 100/0 and 80/20 (v/v), while the overall concentration of PCL in the solution was maintained at 12 wt%. Polylactic acid was dissolved at a concentration of 4 wt% in a solvent mixture of chloroform and DMF.

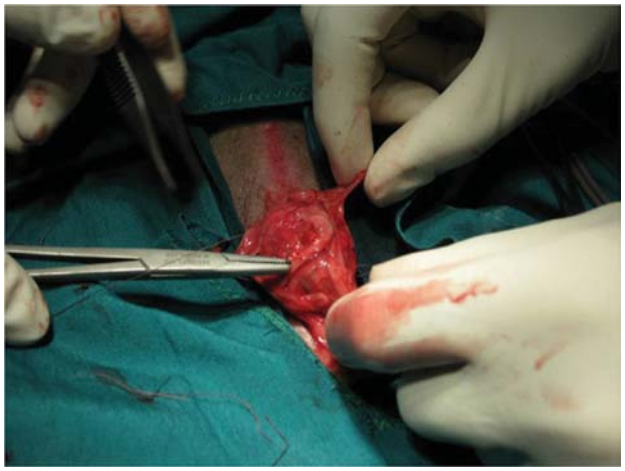

Figure 1. A single layer of continuous interlocking sutures with 4-0 polyglycolic acid was used for the anastomosis of PCL/PLLA to the bladder of the third dog.

A variable voltage power supply was used for electrospinning. Two 10 mL syringes were used to stock each of the prepared solutions. The electrospinning setup utilized in this study consisted of three syringes, a ground electrode (stainless steel drum, with outer diameters of 3 and 5 mm, and the length of 10 cm), and a high voltage supply (25 kV). The PCL and PLLA solutions were delivered with a syringe pump (SP-500; JMS, Tokyo, Japan) at a flow rate of 1 mL to a 20-gauge syringe nozzle through PE extension tubing. The needle tip could move in restricted distance along the direction of the deposition area; thus, gave the capability of having a uniform mat. A voltage in the range of 15 and 30 volts was applied to the solution and the jet emerging from the needle to the drum collector. The collecting surface consisted of a cylindrical stainless steel collector rotating at 2300 RPM. The collector was located in a fixed distance (15 cm) from the needle. The process time for samples considered from 0 to 10 minutes and morphological investigation was 1 minute, while for in-vitro assessment, in-vitro the scaffold was produced in about 2 hours. The samples were then washed 3 or 4 times in sterile water and immersed in 70% ethanol overnight for elimination of bioburden. In order to increase hydrophilicity, oxygen plasma treatment was performed. The bare materials were exposed to oxygen plasma at 13.6 MHz for 5 minutes using a

diener electronic plasma device. Scanning electron microscopy (SEM) observation morphology of the electrospun PCL nonwoven and PLLA mat was ob-© Tescan, served with scanning electron microscopy (SEM, Vega, Cranberry Twp., PA, USA) after sputter coating with platinum. The diameter and the distribution of the diameter of electrospun PCL nonwoven and PLLA mats were measured using image analyzing software. Nonwoven fabric samples with proliferated cells were fixed in a 2.5% glutaraldehyde solution in phosphate-buffered saline (PBS). After rinsing and dehydrating in sequentially increasing ethanol solutions to 100% ethanol, the dehydrated samples were dried in a critical point drier (Christ GAMMA 2-16 LSC) and observed by SEM after sputter coating with platinum.

Anterior bladder wall incision was made, and the cutting margin was sewn to the square patch of nanofibrous scaffold (3 × 4 cm) using vicryl suture and finally omental wrapping was done.

Immunohistochemistry and Histology (Cell Morphology)

For immunostaining, the samples were washed twice with PBS and fixed by 4% paraformaldehyde for 24 hours at the temperature of 4 ºC. These cells were permeabilized and blocked in PBS containing 0.2% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA; Cat No.T8787) and 10% goat serum for 10 and 30 minutes, respectively. Thereafter, the samples were incubated in primary antibody diluted in 0.5% bovine serum albumin (BSA) at 37 ºC for 1 hour. The antibodies used in this study were Desmin (Santa Cruze, sc-14026), pan- cytokeratin (Santa Cruze, sc-15367), and alpha-smooth muscle Actin (Sigma, A5228). At the end of the incubation time, the cells were washed twice with PBS + 0.05% Tween 20 and incubated with the fluorescein isothiocyanate conjugated anti-rabbit IgG (FITC; Sigma, F1262) and anti-mouse IgG (FITC; Sigma, F9006) diluted in 0.5% BSA for 60 minutes at 37 ºC. After being washed twice with PBS + 0.05% tween 20, the specimens were examined under fluorescence microscope (CKX41, Olympus, Japan). Furthermore, for histological examination, the specimens were washed twice with PBS, and fixed by 4% paraformaldehyde for 24 hours at 4 ºC. The samples were then dehydrated through a series of graded alcohol solutions and xylol, and then, were embedded in paraffin. The paraffin-embedded specimens were sectioned perpendicular to the culture surface at 5-µm thickness. The sections were attached to poly-l lysine coating glass slides and placed at 60 ºC in an oven for 12 hours, dewaxed in xylene, and stained with Hematoxylin and Eosin.

Scanning Electron Microscopy

The specimen were prefixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide for 3 hours at room temperature, and then, dehydrated through a series of graded alcohol solutions. Once dried, the samples were mounted on aluminum stubs, sputter-coated with gold-palladium (AuPd), and viewed by using SEM (Tescan VEGA-II, the Check).

RESULTS

As demonstrated, this polymer had high interaction with cells. Hematoxylin and Eosin staining of PCL mat after 3 months of implantation showed bladder cells migration and infiltration to PCL mat plus tissue regeneration. Muscular bundles and urothelial layer were formed.

Macroscopic view of the graft site in the bladder of the first sacrificed dog showed intraluminal encrustation. Clinical degradation was not observed in the first dog; therefore, we increased the fol-

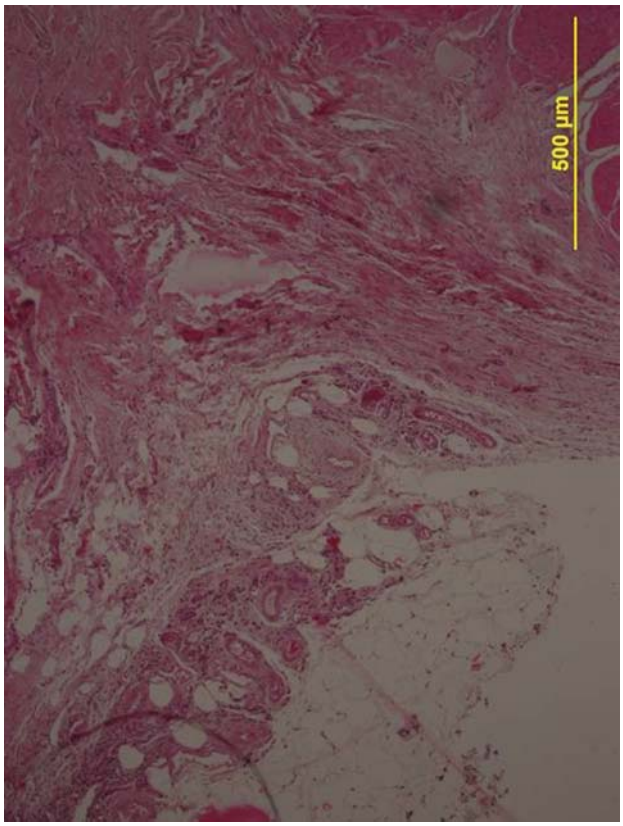

Figure 2A. Hematoxylin and Eosin staining of the harvested graft site of the second dog after 4 months of PCL implantation.

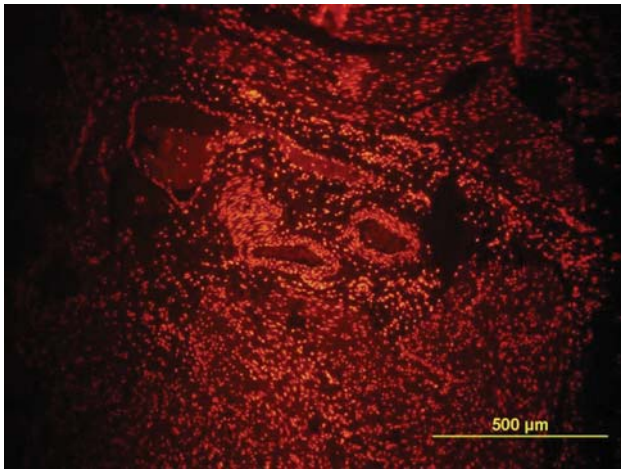

Figure 2B. The immunohistochemical appearance of the harvested graft site of the second dog after 4 months of PCL implantation.

low-up period upto 4 months for the second dog. Hematoxylin and Eosin staining of harvested graft site (PCL) of the second dog, 4 months after implantation, showed proper muscle and urothelial cells migration and infiltration into the mat (Figures 2A and 2B). Again encrustation was observed in intraluminal view of the bladder (Figure 2C).

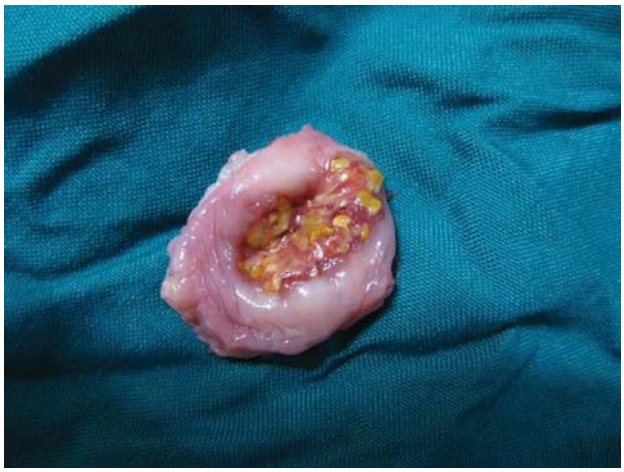

Figure 2C. Encrustation was observed in intraluminal view of the bladder after implantation of PCL.

Although the same experiment with both PCL and PCL/PLLA mats showed the same histological, immunohistochemical, and SEM results, macroscopic view of the bladder in the later case after 4 months revealed normal mucosal appearance, and encrustation could not be detected (Figures 3A and 3B). Therefore, it seems that in our experiments, PCL/PLLA demonstrated better clinical results.

DISCUSSION

The introduction of alternative tissue regeneration technique by using unseeded synthetic polymers has caused enormous controversy. Pattison and colleagues demonstrated that the possible etiology of limitations of this experience, including infection, toxicity, and biocompatibility problems, was related to the micron size of these polymers; thus, these particles could not regenerate the new tissue from the surrounded native cells.[19] In-vitro study by Vance and associates demonstrated that the number of fibroblast cells decreased on mi-

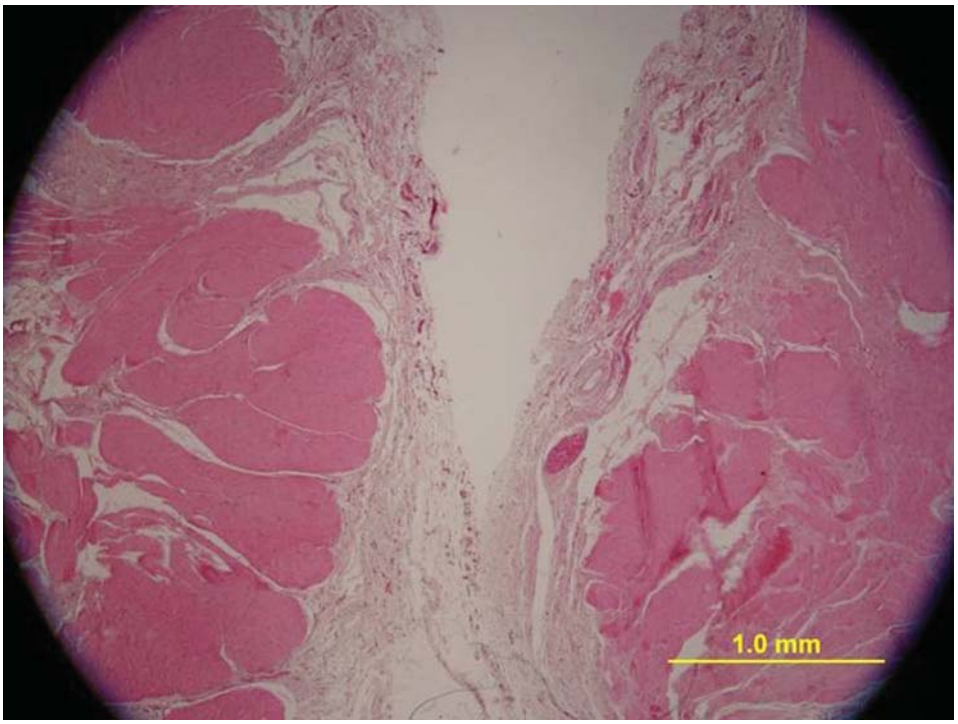

Figure 3A. Hematoxylin and Eosin staining of the harvested graft site of the third dog after 4 months of PCL/PLLA implantation.

cron size polyether urethane (PU), PCL, and polyglycolic acid (PLGA) polymers prepared with NaOH, and finally concluded that micron roughness surface had a negative impact on fibroblast cells growth;[20] hence, modification of these polymers to nano dimensions gained more attention. In comparison to the afore-mentioned micron particles, nano-dimentional materials (< 100 nm) have completely different cellular response. Thapa and coworkers presented the in-vitro study that revealed the enhancement of proliferation and adhesion of the bladder smooth muscle cells on nano-structural complexes, such as PLGA and PU films. Likewise, they showed that cell growth increases over the extended periods of 1, 3, and 5 days after using those nano-structural polymers.[21] Another in-vitro study revealed that cellular adhesion on nano-dimensional polymers (PLGA-PCL-PU) improved significantly in comparison with traditional micron size polymers.[22] Pattison and colleagues produced a nano-rough surface of three dimensional PLGA in the laboratory environment successfully, and then, cellular growth, adhesion, and protein production improved with these particles in comparison to the micron polymers. They recommended that using nano-rough surface of three dimensional PLGA scaffolds may be a proper medium for regeneration of the bladder wall cells in in-vivo environment.[23] Harrington and associates reported their in-vitro experience of implantation of multipotent stem cells on nano-dimensional structure[24] and the other similar studies explored the interesting aspects of using these polymers with two different approaches, including bottom-up (self-assembling nanofiber system was used to culture neural stem cells)[25] and top-down (differentiation was solely controlled by nanotexture size).[26]

Allogenic matrix with seeded cells used in the canine model brought about more noticeable results than the acellular matrix[27] and significant

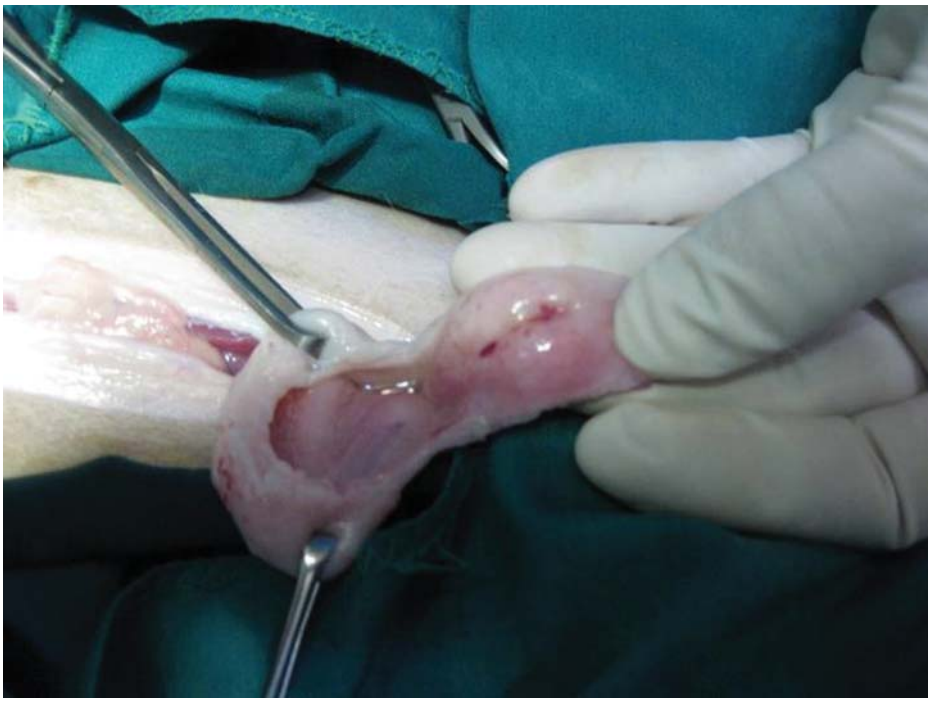

Figure 3B. Macroscopic view of the bladder after 4 months showed normal mucosal appearance, and encrustation was not detected.

improvement of regeneration of the bladder wall cells was observed on scaffold with seeded cells against unseeded (100 times versus 30 times in functional capacity).[28] Domingos and coworkers revealed that the latex biomembrane as a biocompatible agent can be used in a rabbit model for the bladder augmentation successfully and this material promoted epithelium and muscle regeneration with proper clinical and histological outcomes. The transitional epithelium continuity of the host bladder tissue on the patch area and the well-organized muscle layers were detected on the 9th day.[29] The other study demonstrated successful experience of using salt-modified and collagen-coated PCL scaffold in micron dimensions for the bladder wall grafting in 16 rats. Two months after grafting, proper epithelialization and growth of smooth muscle cells were detected.[30]

In this study, we explored the feasibility and safety of PCL and PCL/PLLA scaffolds in nano dimensions in our canine model (an in-vivo study) as a bioavailable substrate layer in reconstruction of the bladder and regeneration of the epithelium and smooth muscle cells. Further efforts are required in future to evaluate the results of cellular seeding on this nano scaffolds that may revolutionize the bladder tissue engineering.

CONCLUSION

All three implantation models showed the same IHC and SEM results; however, only the PCL/PLLA model has come up with desirable clinical results.

To the best of our knowledge, this is the first report that examines the PCL and PCL/PLLA scaffolds in nano dimensions in a big animal model for the bladder tissue engineering. However, further studies by using other nanopolymers may advance the results of tissue engineering in organ reconstruction in the future.

CONFLICT OF INTEREST

None declared.

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