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# In-vivo regeneration of bladder muscular wall with whole decellularized bladder matrix: A novel hourglass technique for duplication of bladder volume in rabbit model

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ABSTRACT

*Objective:* To determine histological aspects of decellularized bladder graft to achieve a double-sized bladder by novel hourglass technique; using rabbit models.
*Methods:* Sixteen rabbit bladders were decellularized and underwent laboratory investigations. After making a laparotomy incision and exposure of bladders in another 16 rabbits (partial detrusor myomectomy), they were separated into two groups. The fundus of the decellularized scaffold was anastomosed to the fundus of the native bladder via the serosal layer, and the omentum and a double-J stent were placed in the decellularized bladder by no direct contact with the urine (Group A, $n=8$). In group B ($n=8$), the bladder was augmented applying the decellularized bladder that was in contact with the urine. After 6 months, the omentum was brought out of the neck of the engineered bladder and the anastomosis was opened. Biopsies were taken at 1, 3, and 9 months postoperatively.
*Results:* Cell removal with preservation of extracellular matrix structure was confirmed in decellularized bladders. Histological examination after 1 month demonstrated few cells at the border of the grafts. After 3 months, the region of the graft was indistinguishable from the natural bladder with continuity of transitional epithelium of natural bladder on the decellularized grafted scaffolds. The organization of muscle layers was similar to native bladder muscle layers after 9 months. IHC staining markers were highly expressed after 9 months. Interestingly, bladders had a high fibrosis grade in group B compared with hourglass technique.
*Conclusion:* We confirmed that decellularized bladder may be a reliable scaffold and viable material for bladder augmentation.

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The urinary bladder is a complex organ and many clinical conditions can cause conditions requiring bladder augmentation or use of regenerative techniques and scaffolds [1].

Bladder augmentation has the ability to protect the upper urinary system by reducing the high bladder pressure that develops in patients with neurogenic bladder [2]. Bladder augmentation is the technique of choice in the treatment of the noncompliant bladder that is refractory to conservative management [3].

Augmentation cystoplasty using gastrointestinal segments is associated with numerous side-effects, such as mucus production, bowel obstruction, stone formation, fibrosis, leakage, and metabolic disturbance [4]. The recent application of the porcine small intestinal submucosa (SIS) and the bladder acellular matrix graft (BAMG) in several experimental studies demonstrated that they act as biodegradable materials that allow the regeneration of urothelial and smooth muscle [5,6]. However, SIS and BAMG are xenogenic materials and require highly developed technique preparation. The ideal material used for bladder augmentation should have surgical viability and minimal metabolic and urological drawbacks.

In view of these drawbacks, tissue engineering technology in combination with surgical construction has been developed with promising outcomes [7,8]. Owing to pressing needs of clinics for alternatives in bladder augmentation and the limits of current biomaterials used in bladder augmentation, this study aims to apply whole decellularized bladder scaffolds for a double-sized bladder autoaugmentation in rabbit models. We used a novel hourglass technique in the purpose to assess the feasibility of this strategy

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as a supplement or replacement for currently used bladder biomaterials.

## 1. Methods

### 1.1. Ethical committee permission

All the animals' procedures were performed in compliance with the regulations of Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council (NIH Publications No. 8023, revised 1978).

### 1.2. Decellularization technique

Urinary bladders were completely harvested from 16 rabbits and underwent a decellularization process. After rinsing the tissues with an antibiotic cocktail and distilled water, 2% sodium dodecyl sulfate (SDS) was applied for 6 h. Then, the bladder tissues were soaked in Triton X-100 for a period of 4 h. In the last stage, the bladders were washed with phosphate-buffered saline (PBS) for 2 h. All of the previous steps were executed under continuous shaking at 70 rpm. After the last step of the decellularization process, bladder scaffolds were evaluated for confirmation of decellularization. The tissues were stored for further implantation process.

### 1.3. DAPI staining

Natural and decellularized bladder scaffolds were stained with 5 mM stock solution of blue-fluorescent 4',6-diamidino-2-phenylindole (DAPI) that was diluted with 30 nM with PBS. Afterward, 300 μl of DAPI solution was added on each slide, incubated in a dark room for 30 min, rinsed in distilled water, and evaluated with an inverted fluorescence microscope (Diaphot 200; Nikon, Tokyo, Japan) in order to visualize dsDNA.

### 1.4. Scanning electron microscopy (SEM)

Both natural and decellularized bladder tissues were fixed in 2.5% glutaraldehyde. After rinsing them in 0.1 m PBS, they were dehydrated in graded ethanol–water series (Merck, Germany). Dehydrated specimens were dried overnight in a desiccator. Dried tissues were mounted on aluminum stubs and sputter-coated with gold–palladium (AuPd) by the application of a Gatan ion beam coater (Gataninc. Japan). They were then viewed with a field emission SEM (FE-SEM; JSM-6340F, JEOL, Tokyo, Japan) with an accelerating voltage of 10 kV. A working distance of 8 mm was used (S3500N; Hitachi High Technologies America) to analyze the structural features of the scaffolds.

### 1.5. Mechanical properties

The tensile properties of decellularized and normal bladders were characterized; using a universal test machine (Zwick /roll, Germany model Hct 400/25 with 25 KN load cell, software toolkit 1998). The ends of the rectangular bladder tissues ($40 \times 30$ mm$^2$) were mounted vertically on two $1 \times 1$ cm$^2$ mechanical gripping units of the tensile tester. An extension rate of 0.1 mm/s was used in the tensile tests. This investigation was performed at room temperature until the presence of a tear in the tissue. Load-deformation data were recorded and the stress–strain curve of both tissues was drawn by the system. The maximal point of this curve indicates the maximum pressure tolerance.

### 1.6. Surgical technique

Another sixteen New Zealand rabbits were randomly allocated into two groups. After making a laparotomy incision and exposure of bladders in these rabbits (partial detrusor myomectomy), they were separated into two groups. We evaluated the results of bladder autoaugmentation by partial detrusor myomectomy. Rabbits of group A ($n = 8$) underwent the novel hourglass technique. Accordingly, the fundus of the decellularized scaffold was anastomosed to the fundus of the native bladder via the serosal layer without opening the native bladder. A double-J stent was placed in the complete decellularized bladder to avoid further obstruction. This chamber was totally separated from the original bladder and had no contact with the urine. The omentum was placed within the decellularized bladder to induce cell seeding on the decellularized scaffold. After an approximate period of 6 months, another surgery was performed on these animals. The omentum was brought out of the recellularized bladder, the neck of the engineered bladder was closed and the anastomosis was opened to reach an augmented bladder (Fig. 1). In group B ($n = 8$), the muscle of the dome of the bladder and the mucosa was removed and the decellularized bladder scaffold was used for bladder augmentation in the original bladder wall which had direct contact with the urine. Biopsies were taken from the implanted bladder in 1, 3, and 9 months after the first operation.

### 1.7. Histology and immunohistochemistry (IHC)

Tissue specimens of natural and decellularized bladders, as well as biopsies obtained at different postoperative follow-ups, were evaluated to determine the efficiency of the decellularization process in removing cell components and estimate the efficacy of in vivo cell seeding on decellularized bladder scaffolds. After fixing the tissue specimens, the sections were deparaffinized and rehydrated by the application of xylene and a gradient of ethanol, respectively. They were subsequently subjected to H&E, Masson's Trichrome, Sirius Red, Pentachrome, or IHC staining. IHC markers are as follows: α-smooth muscle actin (α-SMA), vimentin, cytokeratin AE1/AE3, CD34, and S100 [all manufactured in Dako (Trappes, France)]. In order to stain type I and III of collagen fibers, 0.1% Sirius Red solution in saturated picric acid was applied for a period of 1 h and washed with 0.5% acetic acid. Connective tissue constituents were stained in five colors; using Pentachrome staining. The sections were observed using a light microscope, photographed by the application of a Nikon digital camera DXM 1200 (Amsterdam, Netherlands), and analyzed with Photoshop 10.0 software (Adobe Systems, Inc., Mountain View, CA, USA) and Image Pro (Image Pro Inc., Boston, MA, USA).

## 2. Results

### 2.1. Macroscopic evaluation of the biopsies

All the animals survived the whole period of our study. We did not observe significant shrinkage, reactive or infectious changes in macroscopic view as compared to the natural bladder tissue at the time of biopsy in animals of group A. However, macroscopic evaluation of the grafted decellularized bladders in animals of group B revealed a noticeable shrinkage in the tissue ($n = 2$, 25%), and in some animals the grafted scaffold was automatically rejected, separated from the bladder wall, and fallen into the bladder cavity just like a foreign body without any leakage in the bladder wall ($n = 3$, 37.5%). Additionally, no intraperitoneal leak of urine was observed owing to the perforation of the augment in none of the groups. This was confirmed by precisely examining the marked augmented bladder in the last follow-up.

### 2.2. Properties of the decellularized construct

Histological evaluations revealed no remaining nuclei; while the ECM matrix was well preserved. According to Trichrome staining, collagen fibers did not degenerate after decellularization techniques. This finding was confirmed in Sirius Red staining in which collagens I and III remained within the thinned, decellularized bladder matrix. Intact

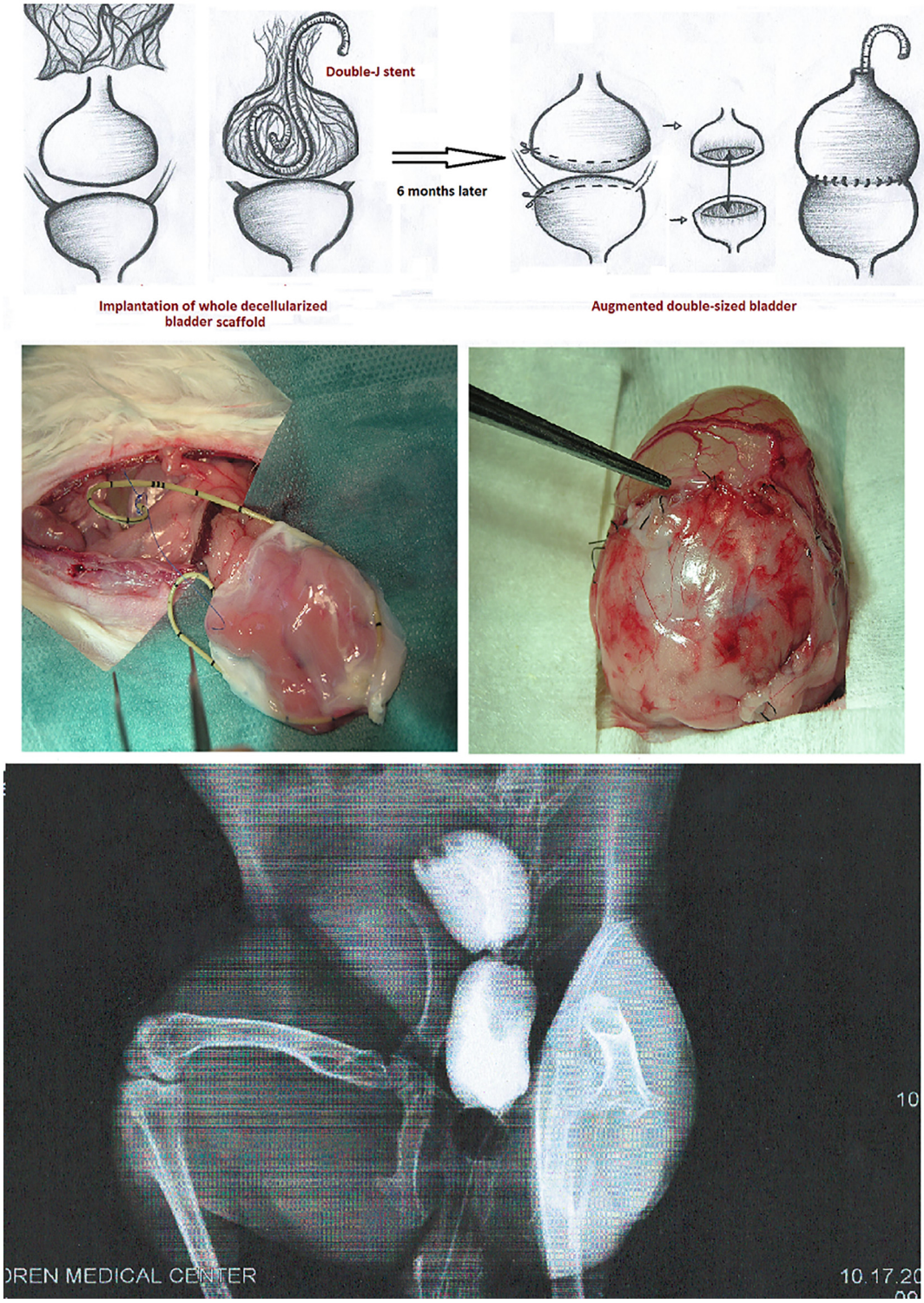


**Fig. 1.** Steps of surgical technique to achieve a double-sized bladder and the schematic view of inverted hourglass technique for blabber augmentation accompanied with the VCUG results after the surgery.

vascular basal membranes were detected within the retained bladder ECM that has a crucial role for further angiogenesis and recellularization (Fig. 2). In mechanical testing, decellularized rabbit bladder samples were highly similar to natural bladders with respect to their stress–strain behavior (Fig. 3, A). The composition and orientation of the bladder ECM were also preserved, while the cells were totally removed (Fig. 3, B).

### *2.3. In vivo recellularization of decellularized bladders*

According to histopathological evaluations in specimens of group A, no fibrosis and inflammatory changes were observed in the biopsies of different follow-ups. The mucosa consisted of transitional epithelium and connective tissue with domed shaped cells on the apical surface. Successful cell seeding with urothelial lining was

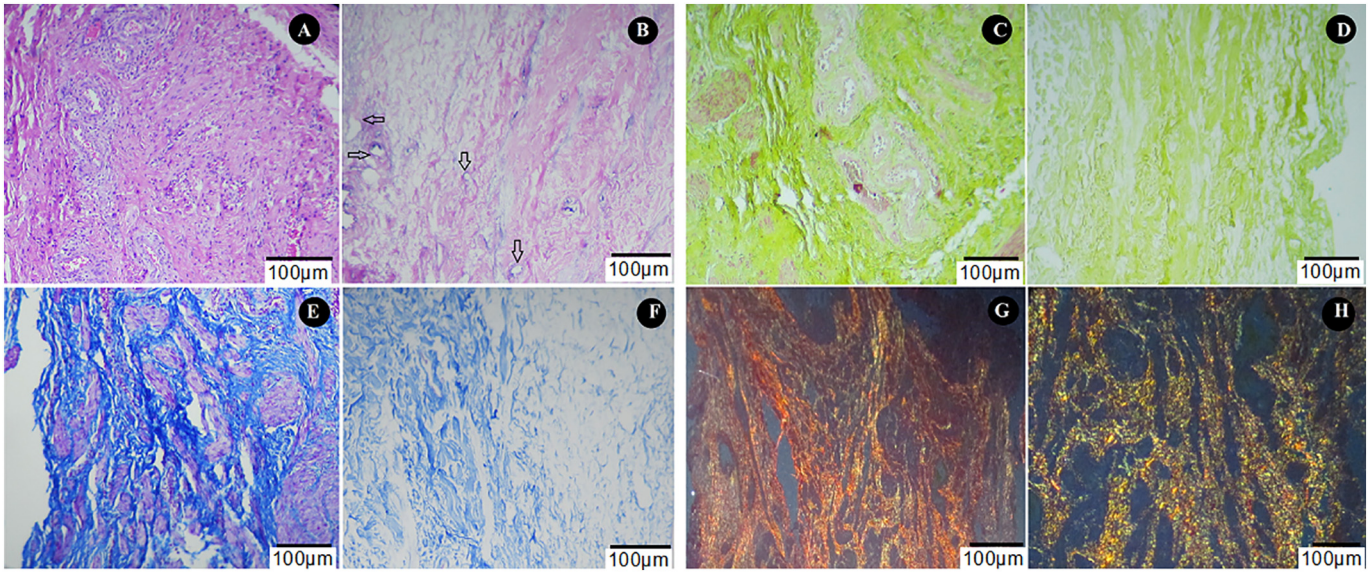


**Fig. 2.** H&E (A & B), Pentachrome (C & D), trichrome (E & F), and Sirius Red (G & H) staining of the natural and decellularized bladder.

observed in all different follow-ups. Biopsies obtained after 3 months of the last step of bladder augmentation procedure were significantly superior to those of the first follow-up in terms of IHC staining for α-SMA, vimentin, cytokeratin AE1/AE3, CD31, CD34, S100. However, there was no significant difference in the histological enhancement of the biopsies obtained after 3 months of operation and the biopsies 9 months postoperatively (Fig. 4/ Table 1). Histopathological evaluation of group B revealed a significantly higher grade of fibrosis in the last follow-up as confirmed by trichrome staining ($p = 0.002$) (Fig. 5).

## 3. Discussion

The histological and immunological results of this preliminary study demonstrate that whole decellularized bladder scaffolds can be successfully used for a double-sized bladder autoaugmentation with noticeable outcomes.

The main aim of the current study was to perform partial detrusor myomectomy for whole bladder grafting without seeding and in situ recellularizations according to our previous report [9] in order to prevent direct contact of urine to the bladder wall ECM and prevent

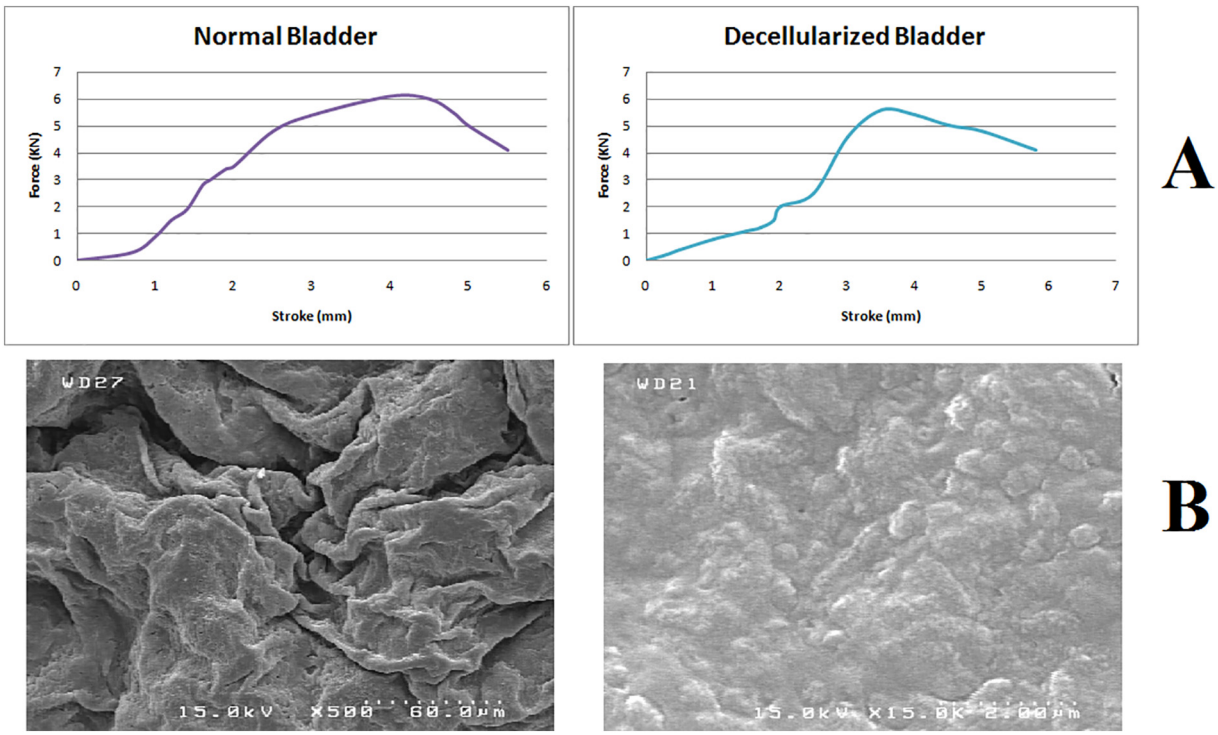


**Fig. 3.** Force/displacement (kN/mm) curve comparing the maximum force of natural and decellularized bladder scaffold (A). Scanning electron microscopy of natural and decellularized bladder tissue (B).

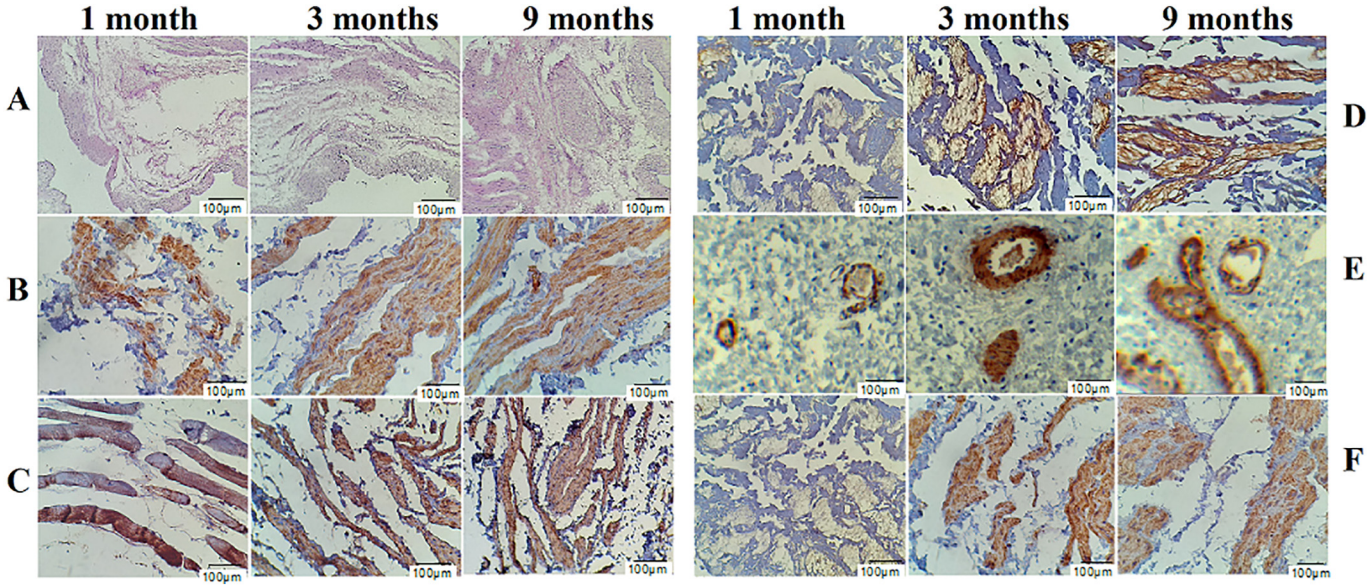


**Fig. 4.** Histopathological evaluations of implanted decellularized bladder of animals in group A at 1, 3, and 9 months postoperatively (×50): H&E staining of tissue-engineered scaffolds (A). IHC staining with cytokeratin AE1/AE3 staining (B) SMA staining (C) vimentin staining (D) CD31 staining (E) and S100 staining (F).

intraabdominal urine leakage. We also aimed to prevent stone formation and compare to the ECM direct contact to the urine as well as shrinkage.

Progressive growth of all components of the normal bladder wall and preservation of mechanical and functional properties of bladder tissue are among the characteristics of an ideal material used for bladder augmentation [10]. The application of biodegradable scaffolds and the injection of isolated cell suspension are among the approaches in tissue engineering. However, experimental transplantation in small animal models is more successful compared to the injection of single-cell suspensions for large tissue reconstruction [3]. Intestinal tissues were used as a gold standard for bladder repair. However, the application of these tissues is associated with considerable long-term complications. Therefore, there is a strong need for alternative techniques, of which tissue engineering and regenerative medicine are considered most promising [11]

In the current study, we attempted to introduce a viable alternative to synthetic materials and other natural scaffolds to support the augmented bladder wall during augmentation using whole decellularized bladder scaffolds. Detrusor myomectomy (bladder auto-augmentation) is a simpler and less morbid alternative to enterocystoplasty. This technique has been also used in this study after making a laparotomy incision and exposure of bladders in rabbits.

In the study of Probst et al. the application of BAMG for bladder augmentation was associated with a high mortality rate (32%) owing to urinary leakage and/or bladder neck obstruction [6]. However, in the current study, we concluded that the whole bladder can be served as a framework of collagen and elastin for the ingrowths of all bladder wall components. In one study in 2017, the performance of a novel asymmetric bilayer chitosan scaffold was compared with conventional bladder acellular matrix graft (BAMG) for bladder augmentation. The results demonstrated the efficacy of the asymmetric bilayer chitosan scaffold in facilitating defect regeneration in the rat model of bladder augmentation [12]. In another study, the feasibility of BAMGs seeded with adipose-derived stem cells (ASCs) followed by intraperitoneal incubation was evaluated for bladder reconstruction in a rat model the results of which showed that ASC-seeded and peritoneally incubated BAMGs promoted the morphological regeneration of the bladder smooth muscle as well as the bladder capacity [13].

Recently, autologous myofibroblast (AM)-silk fibroin (SF) scaffolds with ASCs were used to promote bladder morphological regeneration and improved bladder urinary function. The results demonstrated that AM-SF-ASCs have the potential to repair bladder defects [14].

**Table 1**
Expression of different IHC markers in follow-ups.

| Markers | Grafted decellularized bladder | | | |
|---|---|---|---|---|
| | 1 month | 3 months | 9 months | p value[a] |
| Cytokeratin | 15.75 ± 0.75 | 43.75 ± 0.75 | 49.25 ± 0.75 | 0.03 |
| α-SMA | 22.25 ± 0.50 | 73.75 ± 0.75 | 84.00 ± 0.25 | 0.002 |
| Vimentin | 5.25 ± 0.25 | 35.00 ± 0.25 | 45.25 ± 0.50 | 0.01 |
| CD31 | 17.25 ± 0.25 | 42.00 ± 0.25 | 53.75 ± 0.50 | 0.03 |
| S100 | 10.50 ± 0.75 | 35.50 ± 0.50 | 41.75 ± 0.25 | 0.03 |

Data are means ± SD (% of normal bladder tissue). $P<0.05$ is considered statistically significant. Significant enhancement was found in all IHC markers while comparing the biopsies obtained 3 months after surgery in comparison to those taken at first postoperative month.
[a]The p values are estimated by comparing the values obtained after 1 and 3 months of operation.

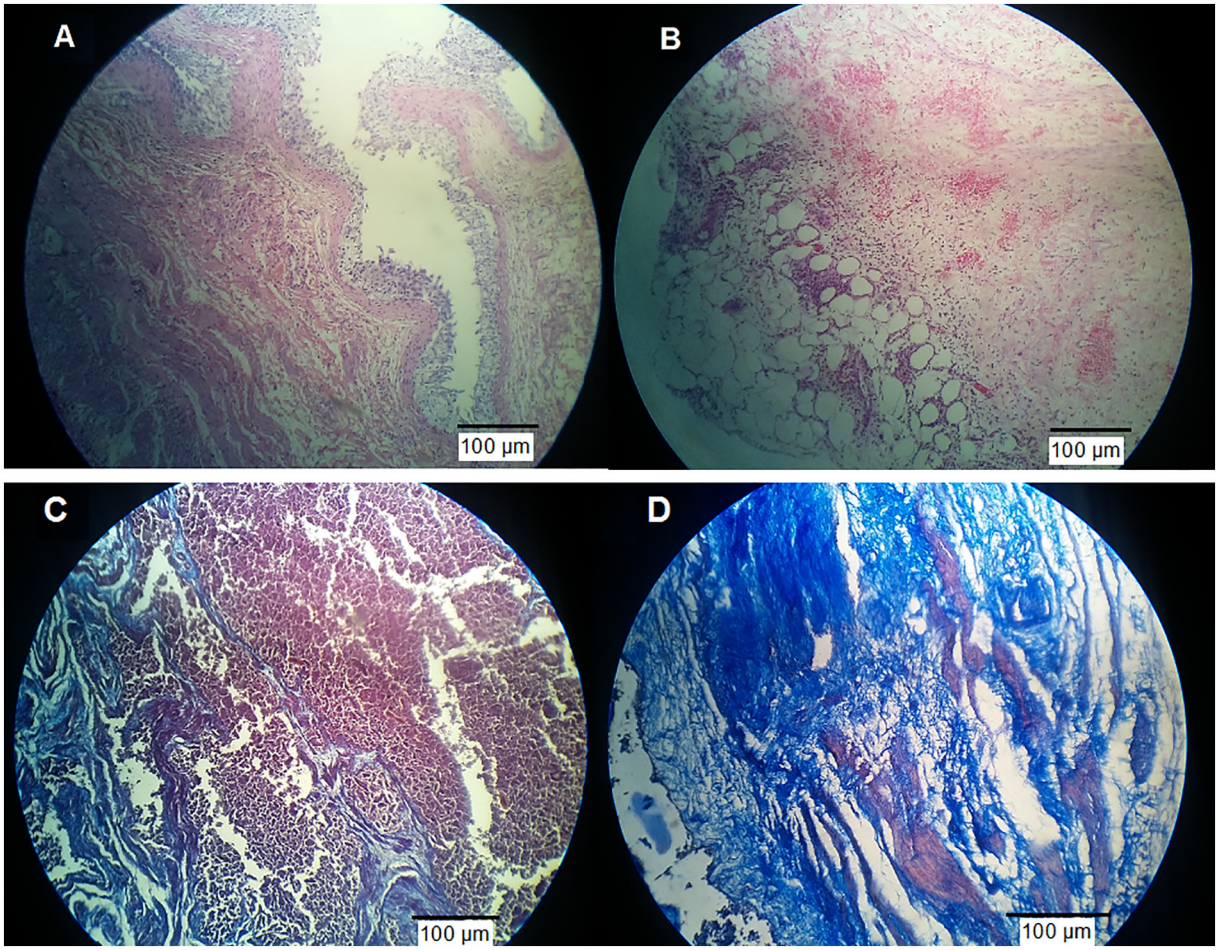


**Fig. 5.** H&E and Trichrome staining of bladders obtained with hourglass technique (A & C) compared with animals of group B with a significantly higher fibrosis grade (B & D).

Although several studies have used ASCs for the purpose of bladder regeneration owing to their multilineage differentiation ability and paracrine role, the low survival rate of ASCs and the difficulty of promoting bladder functional recovery are still unsolved for bladder defect repair. To overcome these problems, we used whole decellularized bladder scaffolds for a double-sized bladder autoaugmentation in rat and rabbit models with remarkable outcomes. In addition, natural acellular matrices may serve as suitable scaffolds owing to the fact that they preserve their original ECM structure and lose their immunogenic characteristics during the decellularization process [15].

In the current study, after successful implantation of the whole decellularized bladder scaffolds, a temporary mild inflammation was present at 1 month postoperatively which subsided gradually during 3 months after the operation. This mild inflammation was completely resolved in 9-month specimens. Owing to natural surgical site inflammation and the stimulation of the host tissue by the grafts, inflammatory responses are expected during the first two weeks after the surgery. In addition, no evidence of graft rejection was observed in any of the animals in group A, the results of which showed that whole decellularized bladder can be used as a biocompatible scaffold for bladder augmentation. By using this hourglass technique, we may be able to produce bladder augmentation and reach a double-sized bladder with intact host bladder mucosa. However, in group B there were contraction and shrinkage of the graft, resulting in the rejection of the grafted tissue in some of the animals owing to the direct contact of scaffolds with the urine. Preservation of the intact urothelial layer over the grafted tissue is of prime importance in order to prevent stone formation. In the current study, there was no evidence of bladder calculus formation.

According to one study in 2018, the application of multiple scaffolds instead of one large scaffold was investigated to improve bladder tissue regeneration and bladder capacity. In addition, implantation of collagen–heparin scaffolds in one bladder demonstrated that tissue was almost indistinguishable from native tissue [17].

In our previous studies, we used different decellularized scaffolds for bladder augmentation with noteworthy outcomes to pave the road for future applications of this natural collagen scaffolds [3,18–21]. However, the use of an extremely large decellularized scaffold may not be a straightforward surgical technique in animal models owing to the high potentials of foreign body formation when exposed to the urine. This novel technique may be useful in successful cell seeding prior to reaching the final double-sized bladder. With the secret of success in this procedure, this hour-glass technique allows the bladder scaffold to acquire new endothelium and muscle before it's connected to the bladder.

The search for the correct material for bladder augmentation is like the search for the Holy Grail and taking this technique to the clinics needs more questions to be answered. However, considering the fact that the only difference between the bladder wall of humans and rabbits is significantly thinner detrusor layers relative to the overall thickness of the bladder wall, this model seems to be a satisfactory experimental representation of this novel technique. In the present study, we focused on the histopathological features of the regenerated bladders and we did not evaluate the functional properties of the reconstructed bladders which are among the limitations of this study. In addition, the duration of the follow up is not sufficient enough to draw a firm conclusion. Considering these promising findings on whole bladder scaffolds in regenerating a full-thickness bladder tissue and adequate smooth

muscle, further studies with larger graft sizes are needed to investigate the impact of this technique scaffolds on bladder volume and capacity.

## 4. Conclusion

Whole decellularized bladder promotes epithelium and muscle regeneration without urinary leakage for bladder augmentation in rat and rabbit models. To the best of our knowledge whole bladder scaffold was not previously applied for bladder augmentation to reach a double-sized bladder as an inverted hourglass technique with intact host bladder mucosa. This study may pave the road for augmentation cystoplasty in patients with a small bladder capacity.

## Declaration of interest

None

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## Disclosure

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