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Augmentation cystoplasty in dogs: A comparative study of different tunica vaginalis grafts

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ABSTRACT

In veterinary practice, numerous urological disorders that cause bladder dysfunction necessitate augmentation cystoplasty (AC). The purpose of this study is to evaluate the dog tunica vaginalis allograft (DTVA), sheep tunica vaginalis xenograft (STVX) and sheep tunica vaginalis decellularized extracellular matrix (STVDEM) as graft materials for urinary bladder (UB) reconstruction following a 45±5% cystectomy model in dogs. In this study, 18 adult apparently healthy mongrel dogs of both sexes were divided into three groups (6 dogs each): the DTVA group, the STVX group, and the STVDEM group. The evaluation of the AC in different groups was carried out using clinical, hematological, serum biochemical, urine, ultrasonographic, retrograde positive cystogram, and histopathological analysis all over the study period of 12 weeks. The dogs in all groups survived the procedures, except three dogs died from both STVX and DTVA groups. The mean bladder capacity indicated that the DTVA and STVX groups had regained 82.22% and 68.62%, respectively, of their preoperative baseline capacity. Interestingly, the STVDEM group's bladder capacity increased to 113.70%. Although histological analysis revealed that the three grafts successfully rebuilt the bladder wall, the STVDEM demonstrated well-organized and well-differentiated epithelial and muscular tissues that resembled, but were not identical to, native UB tissues. As a result, STVDEM is proposed as an ideal and potential acellular graft for UB reconstruction in dogs, whereas DTVA and STVX could be employed in emergencies requiring UB reconstruction.

1. Introduction

Cystectomy is recommended for a number of urinary bladder (UB) affections, including cystic neoplasia, patent urachus excision, iatrogenic injuries, bladder necrosis, recurrent interstitial cystitis, devitalized tissue following bladder rupture, and neurogenic bladder (Granger et al., 2020; Lipscomb, 2018; Milovancev et al., 2020; Sivacolundhu & Withrow, 2013; Tobias, 2011). Furthermore, studies reported that more than 30% of incontinent dogs, particularly those who had undergone ovariohysterectomy, had reduced bladder storage function (Berent & Mayhew, 2017; Burrow et al., 2005). In these cases, augmentation cystoplasty (AC) is proposed after cystectomy as a surgical intervention when medical therapies fail to prevent renal function impairment and pollakiuria (Lipscomb, 2018; Sivacolundhu & Withrow, 2013).

AC has recently replaced urinary diversion due to its lower morbidity (Davis et al., 2011; Watanabe et al., 2011). In addition, it reduces the likelihood of dehiscence and leads to better short-term urine storage (Pozzi et al., 2006). Therefore, the graft material should be biocompatible and capable of acting as a scaffold for the regeneration of UB layers (Brown et al., 2002).

Numerous synthetic materials and biologically derived tissues have been utilized in experimental and clinical settings to reconstruct the UB (Awasum et al., 2019; Elbahnasy et al., 1998; Hansen et al., 1974; Tobias, 2011). Despite the use of several kinds of grafts, gastrocystoplasty and enterocystoplasty are still the gold standard tissues for AC (Abol-Enein et al., 2012; Kispal et al., 2012). The use of the gastrointestinal tract, on the other hand, is linked with considerable morbidity (Kropp et al., 1996; Pozzi et al., 2006; Sabetkish et al., 2014). Therefore, hunting for graft materials that retain the natural transitional epithelium and avoid the need for bowel surgery in bladder repair is still ongoing.

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The best graft material should be available, strong enough to withstand intravesical pressure without rupture or perforation, serve as a scaffold to promote cellular interaction and tissue development, and be biocompatible to avoid immunological complications (Brown & Badylak, 2014).

Previous reports have shown that biodegradable, collagen-based materials that induce native bladder regeneration are effective and successful (Elbahnasy et al., 1998; Kambic et al., 1992). Unfortunately, these materials, such as porcine small intestine submucosa (SIS) (Kropp et al., 1996) and bladder acellular matrix graft (BAMG) (Probst et al., 2000), need extensive preparation or are too expensive for low-cost operations in small animal clinics.

Tunica vaginalis (TV) is the testicular peritoneum, which consists of mesothelium and underlying collagen-rich connective tissue (Liebich, 2019; Wrobel & Bergmann, 2013). The use of TV graft is a simple and low-cost procedure that requires less surgical experience and preparation (Guerios et al., 2020; Ozai et al., 2021). The advantages of TV include its simplicity of harvesting and handling, good tensile and physical characteristics, adequate availability, lack of antigenic properties, decreased foreign body reaction and wound infection, and usage without treatment or preservation (Hua et al., 2019; Pratummintra et al., 2013). Many investigations have confirmed the survival of TV grafts and have verified the transformation of this material into urinary epithelium when implanted in dogs and rabbits' urinary tracts (Calado et al., 2005; Hua et al., 2019; Wongsetthachai et al., 2011).

The aim of this study is to explore the possibility of utilizing dog tunica vaginalis allograft (DTVA) and sheep tunica vaginalis xenograft (STVX) for AC in pet clinics. Furthermore, to the best of our knowledge, this is the first research to use sheep tunica vaginalis decellularized extracellular matrix (STVDEM) as a non-immunogenic acellular collagen that might be a promising potential biomaterial for bladder augmentation in dogs.

2. Materials and methods

2.1. Study design

The study was carried out on 18 mongrel mature dogs of both sexes aged 1-3 years and weighing 15-20 kg at the Department of Surgery, Anesthesiology and Radiology, Faculty of Veterinary Medicine, Suez Canal University. The dogs were given standard dog food that met the National Research Council's (NRC) nutritional standards for dogs (Council, 2006) and free access to water. Prior to the study, dogs were housed for two weeks at the animal's house at the Faculty of Veterinary Medicine, Suez Canal University, for close observation and acclimatization. The animals enrolled in this study were healthy, as indicated by clinical data collected during the acclimatization period; their serum biochemical parameters, urine and total blood count were all within normal ranges. Dogs were divided into three groups: AC of dogs using allogenic TV graft (DTVA) group (n=6), AC of dogs using sheep xenogeneic TV graft (STVX) group (n=6), and AC of dogs using sheep tunica vaginalis decellularized graft (STVDEM) group (n=6). The sheep TV was obtained from the abattoir at Abu Khalifa in Ismailia governorate immediately after slaughter. The adhering fat and fascia were immediately removed and cleaned. The Institutional Animal Use and Care Committee of the Faculty of Veterinary Medicine at Suez Canal University reviewed and authorized all experimental procedures (Approval Number 2021020) in accordance with the ARRIVE guidelines.

2.2. Preparation of the STVDEM

The fresh sheep TV was obtained from mature, healthy rams, and within 30 minutes of harvest, the samples were subjected to a decellularization technique. The decellularization was performed at room temperature following the protocol described by (Sesli et al., 2018). Briefly, the TV samples were treated for two days with a 0.5% sodium dodecyl sulphate (SDS) solution, and then they were subjected to a 1% SDS solution for one day. After washing with phosphate buffered saline (PBS), the samples were treated for 1 hour with a 1% Triton X-100 solution. At the end of each step, the samples were rinsed in PBS. After decellularization, samples were kept at 4°C in PBS with 1% penicillin–streptomycin until the time of surgery on the next day. Hematoxylin & eosin (H&E) and Mallory's staining were used to assess the effectiveness of the decellularization technique and hence confirm the complete absence of all cellular constituents.

2.3. Animal preparation and anesthesia

The dogs were fasted for food and water for 12 and 6 hours prior to the operation, respectively. A prophylactic antibiotic, cefotaxime (Cefotax, EIPICO, Egypt) at a dose of 50 mg/kg intramuscular (IM) was given prior to the surgery. Dogs were premedicated with an IM injection of chlorpromazine hydrochloride (Neurazine, Misr Co. Pharm., S.A.A.) at a dose of 1mg/kg, an IM atropine sulfate (Memphis Pharmaceutical, Egypt) at a dose of 0.04 mg/kg, and an IM injection of nalbuphine HCl (Nalufin®, Amoun pharmaceutical company, S.A.E.) at a dose of 1mg/kg (Clarke et al., 2014). General anesthesia was then induced by intravenous (IV) injection of Propofol at a dose of 2 mg/kg (Diprivan®, AstraZeneca) and maintained by 2% isoflurane (IsoFlo®, Zoetis) and oxygen (Clarke et al., 2014). The ventral abdominal wall was clipped, shaved and prepared for aseptic surgery using Povidone Iodine 10% (Nile Company for pharmaceutical and chemical industries, Egypt).

2.4. Preparation of the dog's TV graft

The dogs were positioned in dorsal recumbence for closed pre-scrotal orchiectomy as described by (MacPhail & Fossum, 2018). After obtaining a TV sheet (Fig. 1a) with a larger diameter than the UB defect, the tunica was stripped of its scrotal fat and fascia. The harvested tunic was put in an aseptic petri dish containing saline 0.9% solution until use (Guerios et al., 2020).

2.5. The AC of dogs using different TV grafts

Prior to the operation, the bladder was emptied using an 8F Foley catheter, which was fixed in place. The partial cystectomy of the cranial 45±5 % of the full thickness of the entire bladder surface area was performed as described by (Tobias, 2011) through a ventral midline approach in bitches and a ventral midline-paramedian approach in males. Following partial cystectomy, AC was performed using different types of TV according to the study groups. The bladder substitute was sutured to the edge of the bladder wall with 2-0 absorbable polyglycolic acid (EGYSORB, Egypt) in watertight continuous sutures (Fig. 1b). The augmented bladder was filled with 20-30 mL of normal saline solution using a previously inserted polyethylene urinary catheter to test the precision of the graft suturing. The abdominal wall closure was routinely sutured as described by (Fossum, 2018).

2.6. Postoperative management

All dogs were injected IM with Cefotax (EIPICO, Egypt) at a dose of (50 mg/kg, every 8 hours for 7 days). The short form of the Glasgow composite pain scale was used to assess postoperative pain in all dogs by an experienced veterinarian who was blinded to treatment (Reid et al., 2007). Rescue analgesia (Nalbuphine HCl, 1 mg/kg) was indicated when the pain score was $\geq$ 6 with consideration of the dog's clinical picture. Nalufin® (Amoun pharmaceutical company, S.A.E.) was injected IM at a dose of (1 mg/kg, every 6 hours for 2 days), while meloxicam (Mobitil, MUP, Egypt) was injected IM at a dose of (0.2 mg/kg daily for 5 days). For wound care, Terramycin skin ointment (Oxytetracycline HCL, Pfizer, Egypt) was applied daily. An Elizabethan collar was placed until suture removal. The skin sutures were removed 10 days after the surgery. The

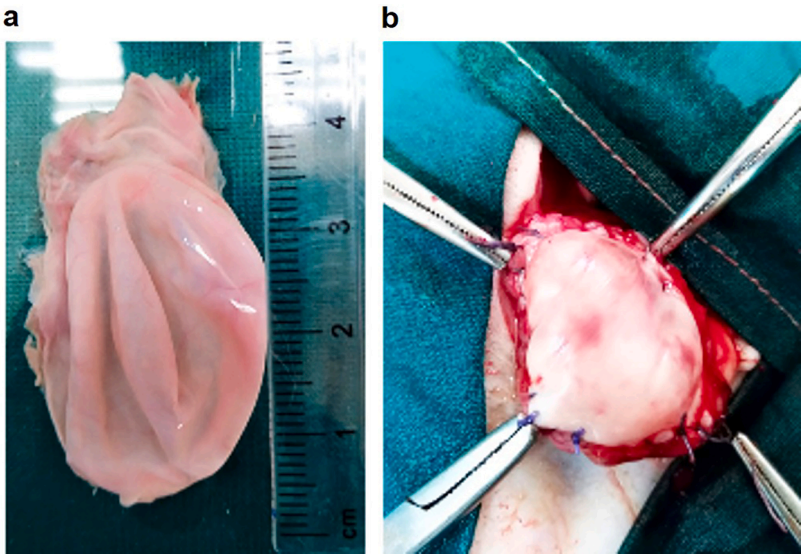

Fig. 1. The appearance of dog TV and UB augmentation. (a) Dog TV after stripping of the scrotal fascia and fat. (b) AC using DTVA graft after partial cystectomy.

urethral catheter was left in the bladder and replaced as needed for 5 days postoperatively.

2.7. Postoperative evaluation

Dogs in the current study were observed postoperatively for general health status, food intake, urination behavior and frequency, as well as abdominal distension. Ultrasonography was performed on the dogs at 2 and 4 weeks following surgery, using a 5 MHz mechanical sector and/or 5:8 MHz linear ultrasound transducers.

The retrograde positive cystogram technique was performed 8 weeks postoperatively as described by (Marolf & Park, 2013). A 20% solution of an organic iodide compound (Urografin 76%, Bayer (Pty) Ltd, South Africa) was slowly injected as a positive contrast agent until full capacity was reached. Radiographic images of the abdominopelvic region were taken for each dog, including lateral and ventro-dorsal views. By the end of the study period (12 weeks), dogs were euthanized with an overdose of sodium pentobarbital IV (Close et al., 1997). UB samples were taken to examine the grafting site macroscopically and microscopically.

2.8. Bladder capacity evaluation

The dogs were placed in a supine position and given light anesthesia. The bladder capacity was assessed prior to the experiment (baseline) and 12 weeks later before euthanasia by measuring the amount (mL) of normal saline needed to completely fill the UB after emptying without resistance using an 8F Foley catheter (Atalan et al., 1998; YÖNEZ, 2018).

2.9. Hematological, serum biochemical, and urine analysis assessments

Whole blood was collected in ethylene diamine tetra acetate (EDTA) tubes and utilized for estimation of complete blood count (CBC) indices. Blood urea nitrogen (BUN), creatinine and C-reactive protein (CRP) were estimated in serum using BUN, creatinine (Abcam, UK) colorimetric kits and canine CRP ELISA kit (Cat. No. 6027, Chondrex Inc., USA), respectively, according to manufacturers' guidelines. Urine analysis was performed on freshly collected urine samples using commercial urine reagent *test* strips (IPee, DFI CO., Ltd, Republic of Korea). Changes in blood and urine were monitored before surgery (baseline), 1, 7, 14, 21, and 28 days postoperatively.

2.10. Histological and Immunohistochemical (IHC) analysis

The UB samples were fixed in a 10% buffered neutral formalin. Standard histological preparation was performed on the sections (6 μm) and stained with H&E in line with (Layton et al., 2019). The staining of Mallory was done to assess collagen, myocytes, and the wall of blood vessels (Layton et al., 2019). The IHC technique was adjusted as previously reported by (Buchwalow & Böcker, 2010). Rabbit alpha smooth muscle actin (α-SMA) (ab5694, Abcam) was used to satin smooth muscle and blood vessel walls. As a secondary antibody, a specific horseradish peroxidase (HRP)-conjugated goat anti-rabbit (ab205718, Abcam) was used. Image J was used to calculate immunostaining densities (Schindelin et al., 2012). Within the 500 μm^2 region of interest, the average intensity of the α-SMA protein signal was normalized to the average control intensity. An Olympus BX41 research optical microscope with an Olympus DP25 digital camera was used to image all H&E and immuno-stained sections (Tokyo, Japan). Finally, H&E stained tissue slices per 0.1 mm^2 unit area were used to determine the number of inflammatory cells in the grafted region.

2.11. Statistical analysis

All quantitative data were expressed as the mean ± standard deviation (SD). SPSS software version 21 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Statistical significance was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. For some experiments, data were analyzed using two-way ANOVA followed by Tukey's post hoc test. The *P*-value was estimated at three levels ($P < 0.05$), ($P < 0.01$), and ($P < 0.001$). GraphPad Prism software version 6 was used to create the graphs (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. The assessment of sheep TV decellularization

The STVDEM became noticeably more transparent. In decellularized graft, the lack of cells and nuclear elements were confirmed by H&E and Mallory's staining, which indicated that the extracellular matrix (ECM) was preserved without collagen degradation. The graft also preserved the architecture of the vascular system with the absence of endothelial cells (Fig. 2).

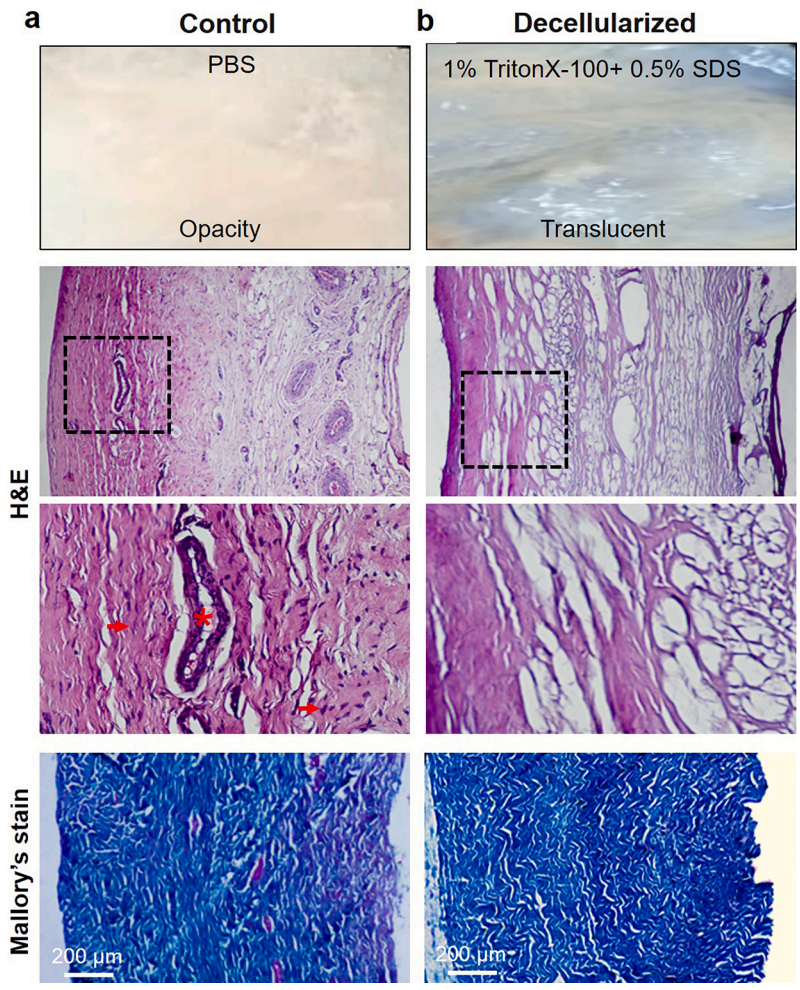

Fig. 2. Gross and histological structure of the sheep TV and underlying fibrous connective tissue before and after decellularization stained with H&E and Mallory's stains. (a) Normal TV was composed of highly oriented collagenous tissue and had white opacity. The TV showed large blood vessels (star) and numerous flat nuclei (arrows). (b) The decellularized graft showed a whitish translucent appearance. H&E and Mallory's staining confirmed that collagen was preserved with no residual nuclei or cells. The zoomed areas are outlined by a rectangular shape. Scale bar= 200 µm.

3.2. Clinical findings

The postoperative clinical follow-up revealed that 14 dogs voided turbid blood-tinged urine via the catheter until the 5th post-operative day, whereas 4 dogs in the STVX group remained until the 18th post-operative day. In the DTVA and STVX groups, dysuria was observed in 3 and 4 dogs, respectively, which disappeared later in one dog from each group. Meanwhile, the other two dogs in the STVX group died on the 10th and 14th postoperative days because of postoperative infections induced by graft rejection, as revealed by postmortem findings. On the 16th postoperative day, one dog in the DTVA group died of septic conditions. The urine frequency gradually returned to the preoperative baseline until the 16th postoperative day, while the remaining dogs in the STVX group had pollakiuria until the 34th day. In any of the dogs that survived, there was no evidence of infection, graft rejection, calculi formation, or urine extravasation.

3.3. Evaluation of the bladder capacity

All dogs' bladder capacity and compliance improved to various degrees following AC. By the end of the experiment, the DTVA and STVX groups had recovered 82.22% and 68.62% of their preoperative baseline capacity, respectively. Surprisingly, the STVDEM group's bladder capacity increased from the preoperative baseline to 113.70%, with no significant difference between the groups and the preoperative bladder capacity at ($P < 0.05$) (Fig. 3a).

3.4. The findings of the hematological, serum biochemical, and urine analysis

The hematological results showed a slight transient reduction in red blood cells (RBCs) indices following surgery. Apart from the elevation of leucogram in all groups, other hematological parameters were non-significantly ($P < 0.05$) altered during the experimental period (Table 1). Serum creatinine and BUN levels were slightly higher in all dogs following AC procedure. Except for the STVX group, all were restored to their preoperative baseline levels by the 7th postoperative day (Fig. 3 b&c). Upon comparing the effects of the implication of the biological scaffolds under study, the results revealed a significant ($P < 0.05$) elevation of serum CRP in all experimental groups one day after the AC. CRP tended to decrease and almost returned to the preoperative level in both DTVA and STVDEM groups. Furthermore, STVDEM group exhibited an earlier return to the baseline value. Meanwhile, STVX displayed a significantly ($P < 0.05$) higher CRP level throughout the experimental period (Fig. 3d).

For the first few days following surgery, urine analysis revealed the presence of hematuria, turbidity, moderate proteinuria, and elevated urine specific gravity. Meanwhile, the STVX group had proteinuria, elevated urine pH, specific gravity, leucocytes, nitrite, and turbid urine for the entire examination period. Other inspected urine parameters displayed no relevant abnormalities (Table 2).

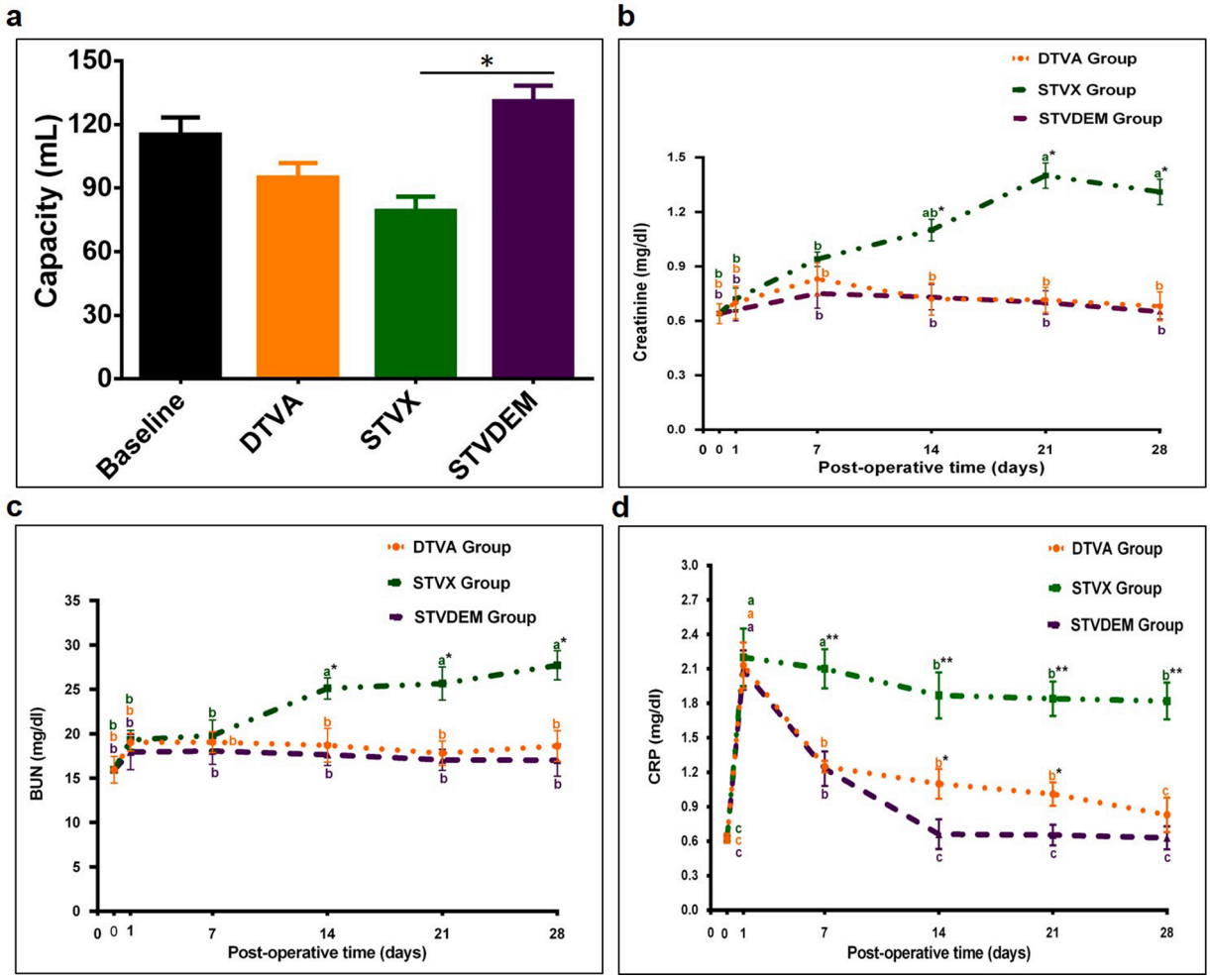

Fig. 3. Bladder capacity and serum levels of creatinine, BUN and CRP after AC in relation to the preoperative values during the study period. (a) Showed a non-significant capacity difference between groups and preoperative values by the end of the study period. However, there was a substantial rise in the capacity of the STVDEM group compared to the STVX group. The quantitative data were presented as the mean ± SD. For multiple group comparisons, statistical significance was evaluated using one-way ANOVA, followed by Tukey's post hoc test. * means $P < 0.05$. (b) Serum levels of creatinine. (c) Serum levels of BUN. (d) Serum levels of CRP. The quantitative data for creatinine, BUN and CRP were presented as the mean ± SD. Multi group comparisons was evaluated using two-way ANOVA, followed by Tukey's post hoc test, where, in the same group, different letters indicate $P < 0.05$, and the same letter indicates $P > 0.05$. At the same time point, * means $P < 0.05$ and ** means $P < 0.01$ between groups.

3.5. Ultrasonographic and cystogram findings

The imaging results showed that there was no indication of urine leakage or diverticulum. Ultrasonographic examination of the DTVA and STVDEM groups 2 weeks after surgery revealed that the UB was oval in shape, not fully distended, with a thickened wall and mixed echogenic intraluminal debris (Fig. 4 a&c). In the DTVA group, one dog had a loosening graft with a large area of adhesion to the dorsally situated surrounding viscera. STVX group, on the other hand, exhibited smaller oval bladders that were not completely dilated with urine and a thicker, uneven, hyperechoic wall. Furthermore, hyperechoic fluid filled the bladder lumen (Fig. 4b).

At 4 weeks, ultrasonographic imaging revealed a slight hyperechoic thickening of the craniodorsal wall of the bladder. The bladders of the DTVA and STVDEM groups restored their native pear shape. Simultaneously, anechoic materials were filled into the bladder lumen, with minimal echogenic material at the ventral wall in the STVDEM group and moderately echogenic material in the DTVA group (Fig. 4 d&f). In the same context, the results of the STVX group were indistinguishable from those at 2 weeks postoperatively, with the exception of a slightly wider bladder lumen and decreased echogenicity of the intraluminal fluid and sediments (Fig. 4e).

The cystogram results revealed no signs of contrast extravasation or fistulas. Bladder volumes differed among dogs. In the DTVA group, the UB displayed appropriate capacity, uniform shape, and smooth configuration with irregularity at the graft area, comparable to the observations in the STVDEM group, with a completely extended and reasonably large capacity (Fig. 5 a&c). In the STVX group, cystogram findings showed a small bladder with an irregularity in the dorsal wall and an extended, uneven contour of the bladder. A diffuse, sometimes irregular, bulging at the bladder's vertex was also observed (Fig. 5b).

3.6. The gross postmortem findings

Gross examination revealed mesenteric and omental adhesions to the bladder wall (Fig. 6 a&b). There were no calculi detected in any of the dogs' bladders when they were opened. Meanwhile, the postmortem findings of the STVX group dog that died on the 10th postoperative day revealed that the graft had been rejected. It detached from the bladder wall and fell into the bladder cavity. One of the dogs in the same group died on the 14th postoperative day, and it was observed that the graft had been floating inside the lumen of the bladder, allowing urine leakage into the abdominal cavity with symptoms of peritonitis. One dog in the DTVA group died on the 16th postoperative day after showing

Table 1
The effect of UB augmentation on the hematological parameters.

Parameter	D0			D1			D7			D14			D21			D28		
	DTVA	STVX	STVDEM	DTVA	STVX	STVDEM	DTVA	STVX	STVDEM	DTVA	STVX	STVDEM	DTVA	STVX	STVDEM	DTVA	STVX	STVDEM
RBCs (10^6/µL)	6.24^{a} ± 0.43	6.36^{a} ± 0.64	6.34^{a} ±0.69	5.77^{a} ±0.39	5.98^{a} ±0.57	5.84^{a} ±0.68	6.45^{a} ±0.52	6.29^{a} ±0.63	6.37^{a} ±0.45	6.30^{a} ±0.71	6.22^{a} ±0.47	6.35^{a} ±0.62	6.34^{a} ±0.42	6.18^{a} ±0.46	6.31^{a} ±0.31	6.38^{a} ±0.46	6.13^{a} ±0.52	6.27^{a} ±0.34
Hb (g/dL)	15.73^{a} ± 1.16	15.92^{a} ± 1.32	16.10^{a} ±1.02	14.28^{a} ± 1.22	14.94^{a} ± 1.17	14.72^{a} ±1.43	15.54^{a} ± 0.92	15.89^{a} ± 1.22	15.42^{a} ±1.37	15.94^{a} ± 1.08	15.29^{a} ± 1.58	15.09^{a} ±1.36	15.91^{a} ± 1.19	15.30^{a} ± 1.41	16.18^{a} ±1.27	15.72^{a} ± 1.29	15.36^{a} ± 1.35	16.07^{a} ±1.07
PCV (%)	48.15^{a} ± 3.53	46.80^{a} ± 3.85	48.72^{a} ±3.71	38.96^{a} ±4.86	39.04^{a} ± 4.61	41.78^{a} ±5.31	49.32^{a} ± 4.05	49.02^{a} ± 2.61	48.06^{a} ±3.22	47.38^{a} ± 4.39	45.02^{a} ± 2.31	49.56^{a} ± 3.06	48.34^{a}±3.33	45.36^{a} ± 3.24	49.51^{a} ± 4.16	48.24^{a} ±3.75	46.28^{a} ± 3.54	49.54^{a} ± 3.16
WBCs (10^3/µL)	6.38^{c} ± 0.52	6.5^{c}± 0.75	6.34^{c} ±0.65	9.78^{b} ±0.43	10.46^{ab} ± 0.83	9.14^{b} ±0.95	10.28^{b} ±0.47	12.36^{a} ± 0.89	6.84^{c} ±0.63	6.92^{c} ±0.79	9.72^{b} ±0.55	6.76^{c} ±0.36	6.75^{c} ±0.43	8.82^{b} ±0.68	6.71^{c} ±0.46	6.49^{c} ±0.93	7.53^{c} ±0.77	6.64^{c} ±0.65

Data represent mean ± SD. Means sharing different superscripts in the same row indicate significant significance ($P < 0.05$)

Table 2
The findings of urine analysis on different study groups.

Parameter	D0			D1			D7			D14			D21			D28		
	DTVA	STVX	STVDEM	DTVA	STVX	STVDEM	DTVA	STVX	STVDEM	DTVA	STVX	STVDEM	DTVA	STVX	STVDEM	DTVA	STVX	STVDEM
Blood	–	–	–	+++	+++	+++	++	++	+	+	++	–	–	+	–	–	+	–
Bilirubin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Urobilinogen	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ketone	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Proteins	–	–	–	+	++	+	+	++	+	–	++	–	–	+	–	–	+	–
Nitrite	–	–	–	–	–	–	–	+	–	–	+	–	–	++	–	–	+	–
Glucose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
pH	6	6	6	6.5	6.5	6.5	7	7	6.5	7	7	6	6.5	7	6	6	6.5	6
Specific gravity	1.015	1.010	1.015	1.030	1.025	1.025	1.025	1.030	1.020	1.025	1.025	1.020	1.020	1.020	1.015	1.015	1.020	1.015
leucocytes	–	–	–	++	+++	+	++	+++	–	+	++	–	+	++	–	-	+	–

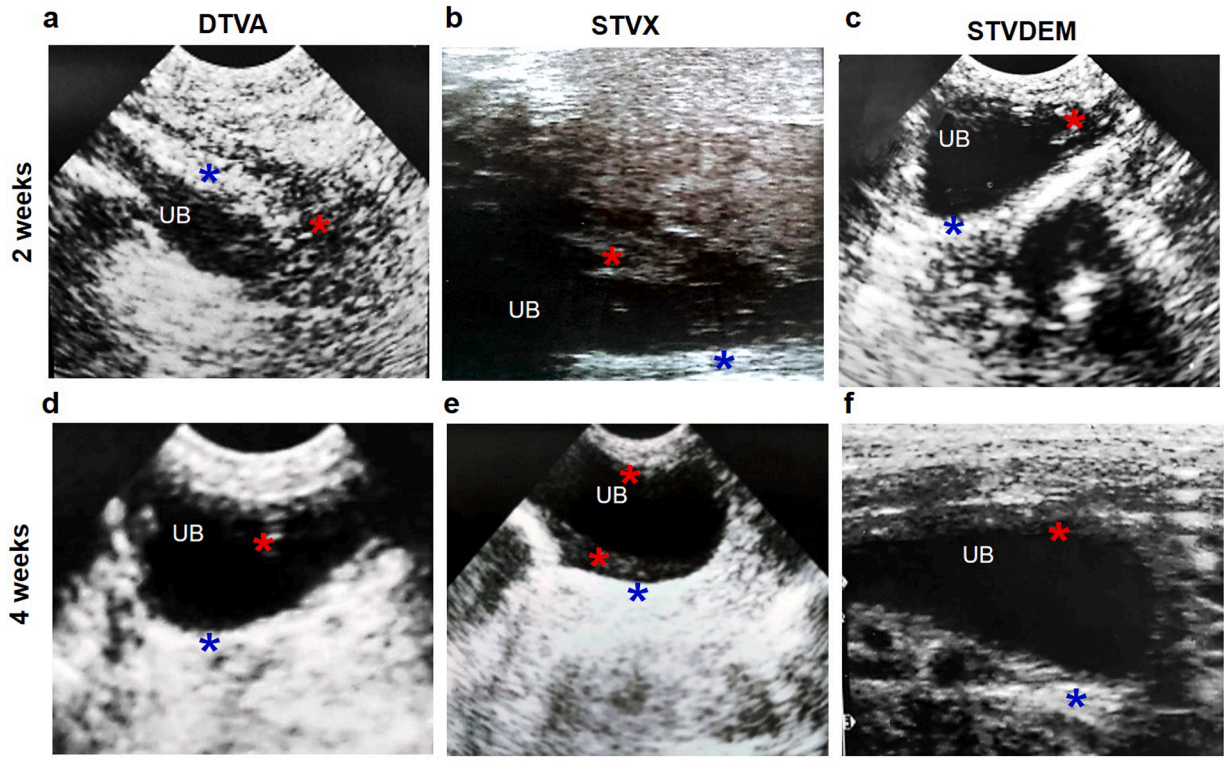

Fig. 4. The ultrasonographic findings of the DTVA, STVDEM and STVX groups at two and four weeks post AC. (a) Modified sector ultrasound, long axis sagittal image of the UB of the DTVA group two weeks postoperatively showing the lumen was filled with moderate echogenic materials (red star) and a hyperechogenic thick band extends along the caudal two thirds of the bladder's long axis close to its ventral side (blue star). (b) Linear ultrasound, long axis sagittal image of the UB of the STVX two weeks postoperatively showing a hyperechoic bladder wall (blue star), while the lumen was filled with hyperechoic fluid (red star). (c) Modified sector ultrasound, long axis sagittal image of the UB of the STVDEM two weeks postoperatively showing a hyperechoic dorsal wall with the graft area adhered to the close viscera (blue star). The lumen was filled with anechoic fluid with clearly distributed hyperechoic sediments close to the ventral wall and the entire neck of the bladder (red star). (d) Modified sector ultrasound, long axis sagittal image of the UB of the DTVA group four weeks postoperatively showing the dorsal wall of the bladder showed normal thickening with hyperechogenicity (blue star). The lumen was filled with anechoic fluid with scantly distributed echogenic sediments close to the ventral wall (red star). (e) Modified sector ultrasound, long axis sagittal image of the UB of the STVX group four weeks postoperatively showing hyperechoic, thickened dorsal wall with heavy hyperechoic adhesion to the surrounding viscera (blue star). The lumen was filled with anechoic fluid with moderate echogenic sediments distributed close to the ventral and dorsal bladder walls (red star). (f) Linear ultrasound, long axis sagittal image of the UB of the STVDEM four weeks postoperatively showing the cranio-dorsal wall of the bladder, showed a hyperechoic graft area with normal thickness (blue star). The lumen was filled with anechoic fluid with a few moderate echogenic sediments close to the ventral wall (red star).

graft necrosis with urine mixed with pus in the UB. All of the other dogs in all groups had good graft attachment to the UB with no signs of leakage, fistulization, or incrustation. Furthermore, the bladders eventually dilate when distended with saline (Fig. 6c). The graft size in the DTVA group had shrunk, leaving only a small round scar at the vertex of the bladder (Fig. 6d), while the STVDEM group showed a non-remarkable scar on the normal bladder (Fig. 6e). The mucosal layer of the bladder replaced the luminal surface of the graft, resulting in a consistent surface that was well integrated with the surrounding tissue and did not bulge. Interestingly, it was difficult to distinguish the STVDEM graft, as it became a small spot with no surface irregularities. On the other hand, the STVX grafts in the remaining dogs of the STVX group were harder than the native bladder wall, with surface irregularities and severe scar formation (Fig. 6f).

3.7. The histology of the augmented bladder by STVDEM and DTVA but not STVX grafts is nearly normal to that of the native bladder

In the present study, three dogs in the DTVA group had disrupted urothelium and were not entirely regenerated as compared to the native bladder, while two dogs showed regenerated urothelium (Fig. 7a&b). Furthermore, islet ball-like lymphoid tissue aggregation was found infiltrating the graft. Concurrently, muscles were organized into discrete, irregular muscle bundles that bore little similarity to the structure of the surrounding native bladders. In the adventitia layer, there was an intense inflammatory reaction, neovascularization, and an excess of collagenous tissue (Fig. 7b). It was noticed in one dog that, the graft was replaced with excessively dense collagen fibrous tissue (Supplementary figure A).

Similar to the DTVA group, three dogs in the STVX group exhibited detached urothelium with many subepithelial collagenous fibrous connective tissue invaded by a localized mass of lymphoid tissue with a few scattered bundles of smooth muscles (Fig. 7c). Additionally, metaplastic calcified tissue appeared as a homogenous acidophilic matrix (Fig. 7c). The fourth dog exhibited more complete urothelium detachment, as with the lack of regenerated muscle fibers in the submucosa. There was extensive infiltration of numerous lymphoid tissue-like and large encapsulated metaplastic calcified materials (Supplementary figure B).

Unlike DTVA and STVX, four dogs in the STVDEM group had multicellular layers of regenerated transitional epithelium at the periphery and throughout the graft area, properly oriented muscle layers, and a normal length of adventitia fibrous tissue with few inflammatory cells and adequate blood vessel invasion (Fig. 7d). However, it was observed that there were some complications in the other two dogs (Supplementary figure C&D).

The total thickness of the UB wall was significantly increased in the STVX and DTVA groups, but remained close to normal in the STVDEM group (Fig. 7e). The detrusor muscle was reduced in the STVX and DTVA

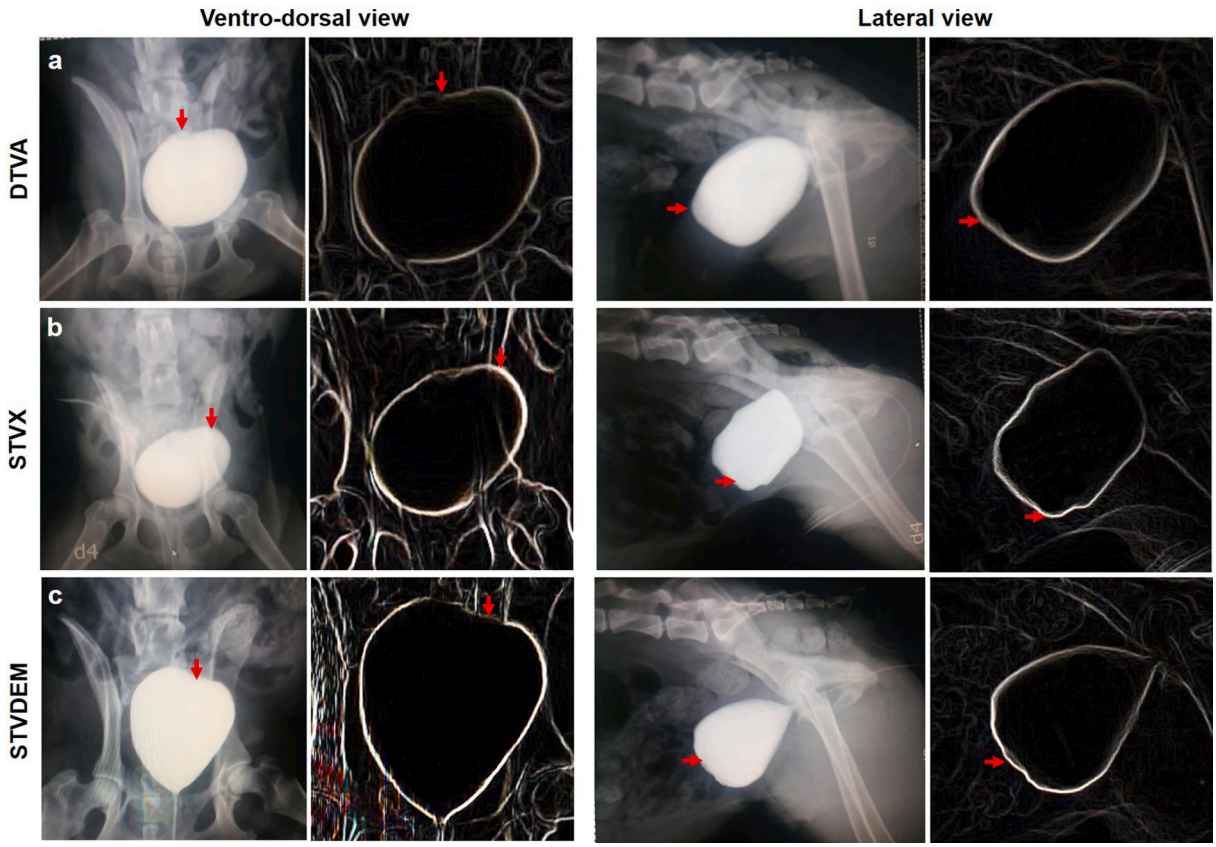

Fig. 5. Retrograde positive cystogram eight weeks after surgery in various study groups (ventro-dorsal and lateral views). ImageJ software was used to clarify the extent of the graft area in the same image. (a) The UB in the DTVA group had a uniform bladder wall and a smooth configuration with irregularity at the graft region (arrow). (b) Cystogram results in the STVX group revealed irregularity in the dorsal wall and bulging at the bladder's vertex (arrow). (c) The STVDEM group had completely engorged UB with a reasonably large capacity and a consistent pear shape, but there was a small depression at the graft regions (arrow).

groups, but in the STVDEM group, it seemed somewhat smaller but substantially regenerated (Fig. 7f). The adventitia layer and surrounding fibrous tissue were significantly increased in the STVX and DTVA groups, but remained unchanged in the STVDEM group (Fig. 7g). There were just a few mononuclear cells and blood vessel infiltrations in STVDEM, compared to greater lymphocyte infiltrations in both DTVA and STVX grafts (Fig. 7h).

In the STVDEM group, α-SMA staining revealed packed smooth muscle fibers and an amply supplied vasculature. In contrast, the DTVA and STVX grafts contained numerous vessels and loose, smaller bundles of intermingled smooth muscle fibers (Fig. 8 a-f). The DTVA and STVX grafts had considerably higher vascular density in the graft region, whereas the STVDEM graft displayed normal vascularity (Fig. 8g).

4. Discussion

The objective of AC is to transform the high-pressure, limited-capacity bladder into a stable environment for urine storage and emptying, therefore minimizing renal function deterioration and maintaining continence (Jednak, 2014). In this context, the current study investigated the utilization and evaluation of the DTVA, STVX and STVDEM for AC in dogs, that mimics clinical settings. Furthermore, this study offers insight for the first time into the use of STVX and STVDEM grafts for cystectomized bladder repair.

The TV was chosen as a viable graft for UB reconstruction in this study based on earlier research that acknowledged the TV as a non-antigenic graft with sufficient availability and lower cost when compared to synthetic material for usage in emergency scenarios (Ozai et al., 2021; Wongsetthachai et al., 2011). STVDEM was presented as a new ECM graft for ease of harvesting and decellularization. The TV allograft and xenograft exhibited adequate bladder wall repair with variable complications in the current research, as highlighted by (Wongsetthachai et al., 2011), particularly with the xenograft. Meanwhile, the use of STVDEM has resulted in the regeneration of the three layers of the UB, which resembled the native one.

A transurethral catheter was left in place until the 5th postoperative day in the current investigation, as advised by (Bakhtiari et al., 2000) to reduce pressure on the suturing line and prevent urine accumulation. In consistent with (Rossetto et al., 2013; Sheta et al., 2014) hematuria was seen in dogs undergoing bladder repair during the first week. These findings were corroborated by urine examinations, which revealed a change in urine colour (red) and appearance (turbid) following AC due to surgical trauma. As a result of blood loss, this was also reflected in lower RBCs indices.

The clinical follow-up in the present study indicated that dogs survived the AC surgery well throughout the study period, which is consistent with the findings of AC using a fascia lata graft in dogs (Ekder & Mahdi, 2021). Regrettably, two dogs in the STVX and one dog in the DTVA group died as a result of graft extrusion and infection, respectively. According to the ultrasonographic and radiographic examinations, no such complications were identified in the remaining dogs in all groups. The same results have been observed in other AC investigations (Akbal et al., 2006; Bakhtiari et al., 2000; Vilar et al., 2004). Urine incontinence reported in the 7 dogs from the STVX and DTVA in the first weeks postoperatively may be due to surgical pain with abdominal muscle strain during urinating. The absence of symptoms afterwards indicates that the graft integration was successful (Ekder & Mahdi, 2021).

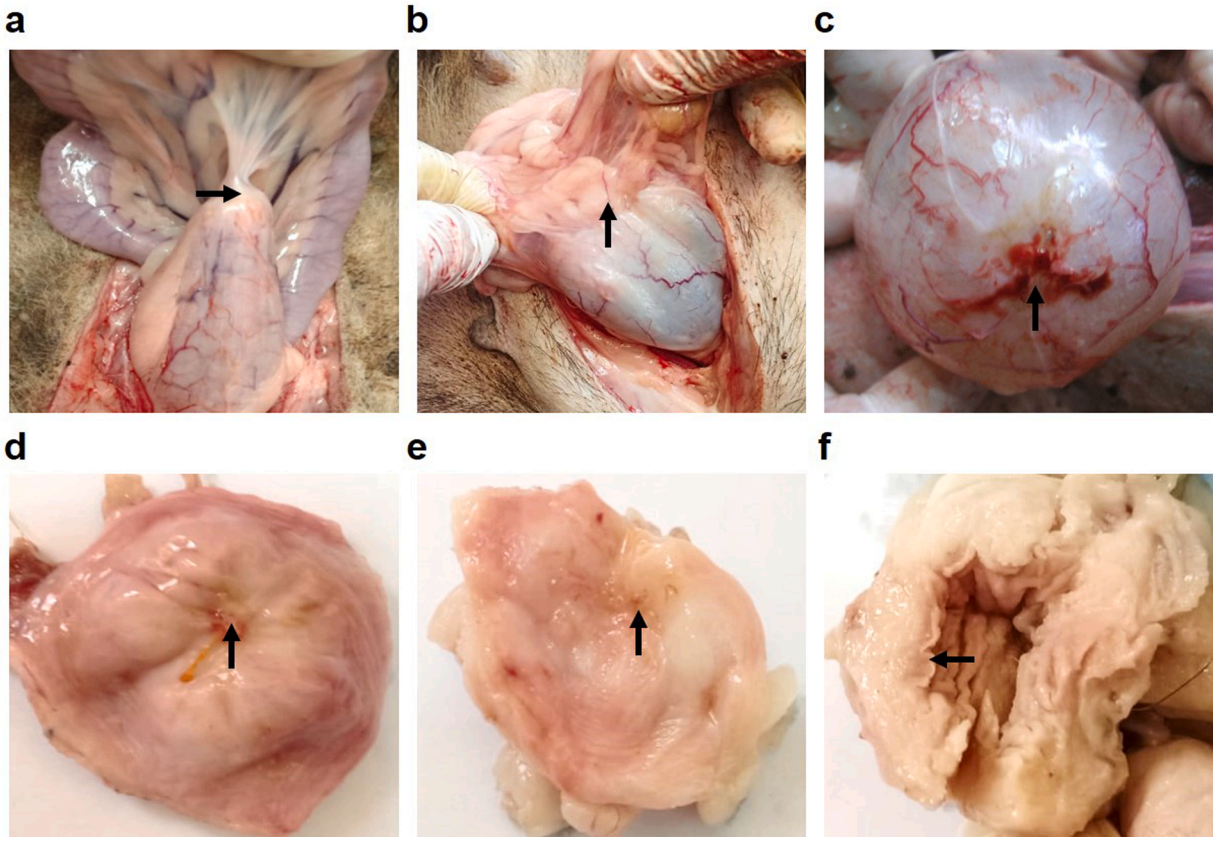

Fig. 6. Postmortem photographs of DTVA, STVX and STVDEM groups 12 weeks post AC. (a) A dog from the DTVA group showed mesenteric adhesion to the graft area (black arrow). (b) A dog from the STVX group revealed omental adhesion to the graft area (arrow). (c) A fully distended UB in the STVDEM group. Note that the graft area is well incorporated into the native bladder with remnants of the graft appearing as a small spot on the vertex (arrow). (d) The DTVA graft (luminal side) showed a small scar at the graft area (arrow). (e) The STVDEM graft (luminal side) showed normal mucosal covering of the graft area without scar formation (arrow). (f) The STVX graft (luminal side) demonstrated thickening and irregularities in the bladder wall (arrow).

In the same context, the observed postoperative increase in serum CRP levels in the experimental groups might be attributable to surgical trauma-induced inflammation (Christensen et al., 2015). Its level gradually declined to preoperative levels in the DTVA and STVDEM groups, thus consolidating promising clinical outcomes (Messias et al., 2020). Eventually, the current study found that AC via STVDEM led to the preservation of normal blood homeostasis and the restoration of urinary system function. However, the persistence of relatively high serum CRP levels in the STVX group, which coincided with elevated urinary nitrite, leucocytes, and pH, strongly suggests the occurrence of an urge inflammatory response to the augmented scaffold material and is directly related to the development of postoperative infectious complications (Alsaif et al., 2021; Liang et al., 2021). The present data clarified that reconstruction of UB using STVX led to impairment of urinary system function. This was also emphasized by the elevated blood WBCs, BUN and creatinine levels in this group. Similar results were observed with gastrocystoplasty in dogs (Muraishi et al., 1992). Contrarily, the DTVA and STVDEM groups reported non-substantial alterations in blood and urine constituents following AC, which was consistent with AC outcomes using other graft materials (Kambic et al., 1992; Rossetto et al., 2013; Sheta et al., 2014).

The TV provides a leak proof graft for UB after AC. Consistent with the findings of BAMG in dogs, the STVDEM showed more capacity than the preoperative level (Probst et al., 2000). Unfortunately, the bladder capacity in the DTVA and STVX groups was lower than in the preoperative values. (Wongsetthachai et al., 2011) attributed the decreased bladder capacity following surgery to shrinkage of the grafted section of the bladder induced by inflammation and fibrosis inside the graft. However, when compared to the preoperative value, the loss in capacity was not significant, and the capacity may have increased over time.

In agreement with prior AC studies (Wongsetthachai et al., 2011; Zhao et al., 2015), there was a varied degree of adhesion between the graft side and the omentum or mesentery, with the STVX graft having the most severe adhesion and the STVDEM having the least. This might be related to the graft's xenogeneic nature (Agishi et al., 1975), as opposed to STVDEM's decellularized nature (Kropp et al., 1996).

Ultrasonographically, bladder wall irregularity and intraluminal debris were seen, especially in DTVA and STVX dogs, indicating postoperative infection and cystitis two weeks after surgery. The same outcomes were outlined previously (Bakhtiari et al., 2000). In contrast, in the majority of STVDEM dogs, the bladder wall was well defined, nearly normal, with distinct layers and a hyperechoic graft region that could be easily differentiated from the surrounding viscera. The bladder appeared to have a normal shape and location after four weeks, especially in the STVDEM and DTVA groups. This was consistent with the findings of AC using amniotic membrane graft (Sheta et al., 2014), whereas dogs in the STVX group had small oval bladders that were not fully distended and had a thickened, irregular wall that was demonstrated as a hyperechoic thickened dorsal wall with heavy hyperechoic adhesion to the surrounding viscera. The same findings were obtained with the bovine pericardium graft for AC in dogs (Agishi et al., 1975). The cystogram findings supported the ultrasonographic findings and indicated that the UB in the STVDEM group had a comparatively large capacity. This owed to the absence of a remarkable scar at the postmortem findings with the presence of multi-cellular layer of a regenerated transitional epithelium at the periphery and throughout the graft area. In addition to, properly orientated muscle layers, and a normal length of adventitia fibrous tissue as denoted in the histopathological

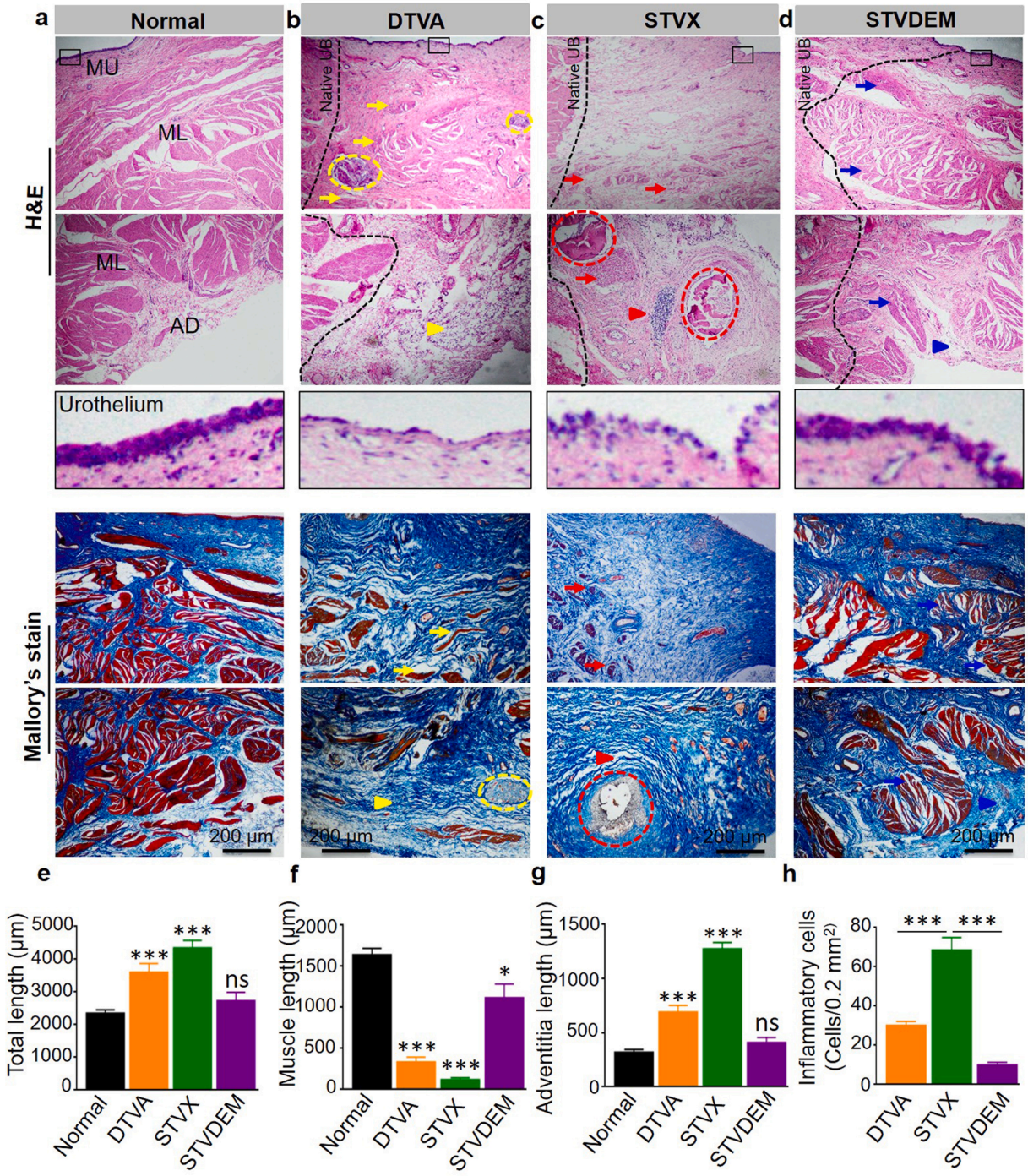

Fig. 7. Histopathological findings of DTVA, STVX and STVDEM groups 12 weeks post AC. Histological sections were stained with H&E and Mallory's stain. (a) Mucosa (MU) with intact 4-5 layers of transitional epithelium (urothelium), muscle layers (ML) in various orientations, and a thin layer of adventitia (AD) were present in normal UB (native bladder wall adjacent to the grafted part). (b) In the DTVA group, three dogs showed disrupted urothelium at the junctional line (rectangular), islet's ball-like metaplastic lymphoid tissue aggregation infiltrated the graft in the contact region and throughout the central graft (yellow circle). The muscles were grouped together into discrete, irregular bundles with little resemblance to the structure of neighboring native bladders (yellow arrow). The presence of an inflammatory infiltration, neovascularization and excess of collagenous tissue was notable and more intense in the AD (yellow arrow head). (c) In the STVX group, two dogs exhibited disrupted urothelium (rectangular) and numerous subepithelial collagenous fibrous connective tissue, which were infiltrated by a focal mass of lymphoid tissue (red arrowhead), metaplastic calcified tissue (red circle) and excessive fibrovascular tissue proliferation in AD. A few dispersed bundles of smooth muscles were present in the adjacent native UB tissue (red arrows). (d) In the STVDEM group, three dogs showed a multi-cell layer of regenerated transitional epithelium at the periphery and throughout the graft area (rectangular), properly orientated muscle layers (blue arrows), and an acceptable length of AD with few inflammatory cells and adequate blood vessels invasion (blue arrow head). Quantitative analysis of (e) the average total length of UB, (f) muscle length, (g) AD length, and (h) the number of inflammatory cells in the study groups compared to the native bladder adjacent to the grafted part. The quantitative data were presented as the mean ± SD. Statistical significance was evaluated using one-way ANOVA, followed by Tukey's post hoc test. *means $P < 0.05$ and ***$P < 0.001$, ns indicated non-significant. Scale bare= 200 µm.

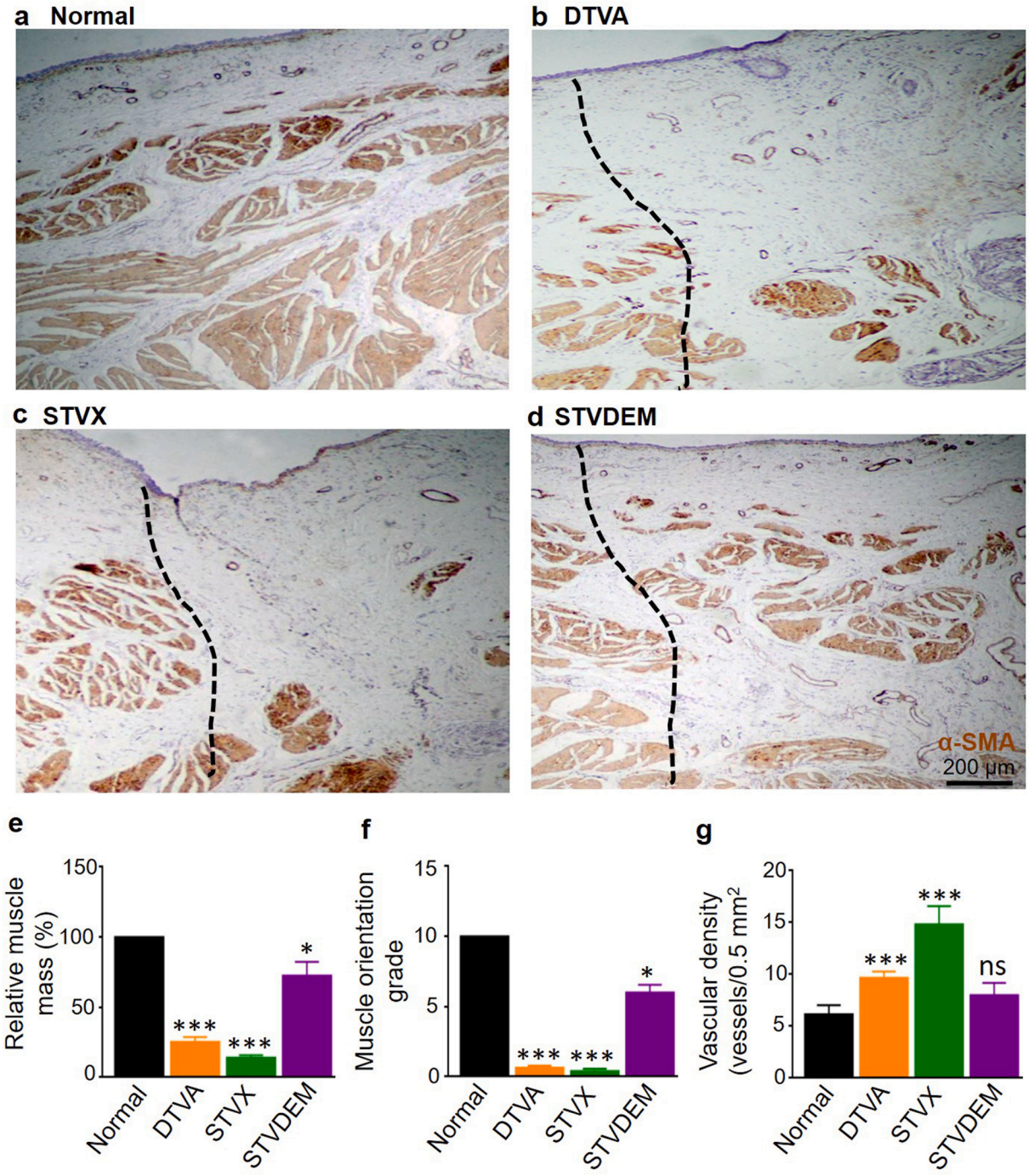

Fig. 8. Representative microscopic images of the UB after AC in different study groups at 12 weeks postoperatively. (a-d) The grafts of different groups were immunoassayed with the anti α-SMA antibody, compared to the normal bladder (native bladder wall adjacent to the grafted part). The α-SMA staining showed abundant smooth muscle layers and normal vascular density in the STVDEM group compared with those of the other two grafts. (e) Quantitative analysis of the average relative muscle mass, (f) muscle orientation grade and (g) the vascular density (vessels/0.5 mm^2 in the study groups compared to normal UB (native bladder wall adjacent to the grafted part). The quantitative data were presented as the mean ± SD. Statistical significance was evaluated using one-way ANOVA, followed by Tukey's post hoc test. *means $P < 0.05$ and ***$P < 0.001$, ns indicated non-significant. Scale bare= 200 μm.

examination, which was consistent with previous research (Iimori et al., 2020).

On cystography, there was restricted filling capacity of the UB in the investigation of (Walker et al., 2002), and the same results were seen in the STVX group, which is associated with calcified metaplasia at the bladder wall. The postmortem and histological examination of the STVX graft revealed that it was harder than the normal bladder wall, with surface abnormalities and extensive scar and fibrous tissue development. The excessive fibrovascular tissue growth and sub-epithelial metaplastic bone (calcified) tissue corroborated these findings histologically (Kambic et al., 1992; Probst et al., 2000; Wongsetthachai et al., 2011). Meanwhile, DTVA and STVDEM groups did not show similar results, which agreed with previous investigation (Kropp et al., 1996). The cystography of the STVDEM group revealed engorged and well preserved UB architecture with no signs of leakage, indicating advanced healing and adaption. Variations in graft preparation and

processing may have contributed to these contradictory results (Wongsetthachai et al., 2011).

In the present study, the STVX graft and one DTVA graft were replaced by scar tissue with cicatricial contraction of the bladder vertex, detached urothelium and absence of muscle regeneration into the fibrous scar. The muscle cell layer stops abruptly at the fibrous scar bladder wall junction. The gross inspection of the TV graft in the remaining DTVA and STVDEM dogs, on the other hand, revealed a well-integrated graft, notably in the STVDEM group. The regenerated urothelium revealed during histological examination of dogs from STVDEM and two dogs from the DTVA indicated that there was no reaction followed by rejection of the implanted graft. Equivalent results were achieved when alternative graft materials for AC were employed (Ayyildiz et al., 2008; Sheta et al., 2014; Wongsetthachai et al., 2011). Furthermore, the STVDEM graft showed well-organized and differentiated epithelium, connective tissues, and a spectacular muscular layer that looked close to the native UB (Kajbafzadeh et al., 2018). The key difference between this remodeled tissue and the native UB was the lesser amount of smooth muscle and the greater amount of collagenous tissue in the graft (Brown & Badylak, 2014; Estrada Mira et al., 2019; Salehipour et al., 2016). The difference in muscle regeneration across TV groups may be related to urothelium regeneration, since bladder smooth muscle does not form when the bladder lacks urothelium (Brown et al., 2002; Iimori et al., 2020).

In preclinical models, comparing different ECM grafts with STVDEM in AC has had conflicting outcomes (Lin et al., 2014; Probst et al., 2000; Sabetkish et al., 2020). The BAMG's applicability was limited due to the complex technique required for its preparation (Ayyildiz et al., 2008; Sabetkish et al., 2014). In agreement with (Sabetkish et al., 2014), the sheep TV was decellularized without the use of any enzymes such as DNase, RNase, or Azide. It was discovered that some of these enzymes were not washed out and remained in the graft, causing teratogenic residues and toxicity. At the same time, STVDM, like the porcine SIS graft, has been shown to induce bladder regeneration of transitional epithelium and smooth muscle while eliciting no immunological rejection (Zhang et al., 2006).

In contrast to the findings of STVDEM, (Rossetto et al., 2013) discovered that the porcine SIS graft differed histologically from the native bladder in terms of the quantity and arrangement of smooth muscle fibers. Furthermore, if the bladder was repaired with SIS alone without cell seeding, it would become permanently contracted. Consequently, the current study evidenced the occurrence of successful bladder AC and restoration of urinary system function in the STVDEM and to some extent, the DTVA groups.

5. Conclusion

This study presented a promising graft for AC in dogs using STVDEM for veterinary clinics. It also proved the biocompatibility and histological outcomes of the STVDEM scaffold without any urinary leakage. Based on the study findings, the DTVA graft showed effective, durable, and agreeable results as a UB substitute in emergencies. Meanwhile, the STVX results were disappointing since the bladders became contracted at the graft site with decreased capacity. More research, particularly in diseased animal models, should be conducted over a longer period of time, as grafting a healthy bladder does not imitate the clinical condition that is eventually being treated.

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Disclosure statement

None of the authors has any financial or personal relationships that could improperly affect or prejudice the content of the article.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical statement

The Institutional Animal Use and Care Committee of the Faculty of Veterinary Medicine at Suez Canal University reviewed and authorized all experimental procedures (Approval Number 2021020), in accordance with the ARRIVE guidelines.

Supplementary figure A: Representative images of DTVA graft stained with (a) H&E and (b) Mallory's stain. The graft was completely replaced with dense collagen fibrous tissue, which was infiltrated with only spares of myocytes at the periphery border of the graft (arrow). Scale bare= 200 μm.

Supplementary figure B: Representative images of STVX graft stained with H&E. (a) A more severe disruption of urothelium (arrow) with no regenerated muscle fiber in the submucosa was observed. (b) Prominent infiltration of multiple lymphoid tissue like and encapsulated metaplastic calcified (star) were present in AD. Scale bare= 200 μm.

Supplementary figure C: Representative images of STVDEM graft stained with H&E. (a) Thickening of urothelium with epithelial metaplasia (arrows) was observed at the periphery of the graft. (b) Towards the center of the graft, the urothelium was reduced to 1-2 layers. Scale bare= 200 μm.

Supplementary figure D: Representative images of STVDEM graft stained with (a) H&E and (b) Mallory's stain. Decellularized tissue did not reach the same level of structure as native tissue. Dense collagen fibrous tissue remained throughout the graft with a few swaps of smooth muscle at the periphery of the graft (arrows). Scale bare= 200 μm.

CRediT authorship contribution statement

Mahmoud F. Ahmed: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Resources. **Elsayed Metwally:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Resources. **Yasmina K. Mahmoud:** Methodology, Formal analysis, Investigation, Writing – original draft, Resources. **Saber M. Abuzeid:** Methodology, Formal analysis, Investigation, Writing – review & editing, Resources. **Mohamed H. El-Daharawy:** Formal analysis, Investigation, Writing – review & editing, Resources. **Mohamed A. Hashem:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.vas.2022.100247.

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