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Clinico-hemato biochemical changes after reconstruction of urinary bladder using autograft and xenogenic EDC cross linked decellularized urinary bladder, pericardium and diaphragm of caprine origin in New Zealand white rabbits

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Abstract
The objective of the present study was to test the efficacy of bladder decellularized urinary bladder, pericardium and diaphragm of caprine matrix graft as cell delivery vehicle and to evaluate the process of bladder regeneration in rabbits. Partial cystectomy was performed on 24 adult New Zealand white rabbits and the defect (1 cm × 1 cm) was repaired with autograft (n=6 group A) and 1% EDC cross-linked decellularized urinary bladder (n=6 group B), pericardium (n=6 group C) and diaphragm (n=6 group D) of caprine origin. The healing was evaluated on the basis of clinical and haematobiochemical observations. In none of the animals signs of graft rejection were observed. The clinical and haematobiochemical observationsdid not reveal any pathological changes. The values of the WBC count, Blood Urea Nitrogen and Creatinine were not changed significantly and found within normal limit.

Keywords: New Zealand white rabbits, autograft, EDC cross linked decellularized urinary bladder, pericardium, diaphragm

Introduction
Severe bladder dysfunction induced by disease or surgical intervention can result in chronic urinary incontinence and increased upper urinary tract pressure leading to irreversible kidney damage. Currently, it has been reported that the treatment of choice in patients with enterocystoplasty the is to increase bladder capacity and lower the storage pressure (Horst *et al.*, 2010) [11]. However, this procedure fails to restore the emptying function and is associated with many complications such as metabolic disturbance, increased mucus production, urolithiasis, infections and even malignant diseases (Nuininga *et al.*, 2004) [16]. In view of these complications, alternative substrates have been investigated with only limited success. Tissue engineering techniques may offer new treatment options for patients with severe bladder dysfunction (Horst *et al.*, 2010) [11]. Tissue engineering (TE) is currently considered a possible solution. Tissue engineering is the combination of biomaterials and bioengineering principles with cell implantation or the directed growth of host cells to develop a tissue or an organ that can substitute native tissue, both in structure and function. The choice of a suitable scaffold for cell delivery and /or the ingrowths of bladder wall components are recognized as one of the key factors that determine regenerative capacities and graft function in augmentation cystoplasty. The engineered scaffold must provide optimal structural integrity to withstand *in-vivo* requirements and appropriate regulation of cell behaviour (Atala *et al.*, 2000) [2]. The homologous and heterologous bladder acellular matrix graft has been demonstrated to serve as a scaffold for the ingrowth of all bladder wall components in rats (Piechota *et al.*, 1998) [17] and pigs (Brown *et al.*, 2002) [4]. bladder acellular matrix graft is collagen-based non-immunogenic membranes derived from homologous or heterologous tissues. BAMG permits the regeneration of native epithelium acting as a scaffold, allowing cross healing of the edges and also promoting angiogenesis and growth of smooth muscle bundles (Sutherland *et al.*, 1996) [25]. In our study, we aimed to evaluate biochemical changes after reconstruction of urinary bladder using autograft and xenogenic diaphragm, pericardium and urinary bladder of caprine origin in New Zealand white rabbits.

Materials and Methods

Animals

A total of 24 adult healthy New Zealand white rabbits of either sex aging between 10-12 months and weighing mean 2.089 ± 0.52 kg were used in this study. The rabbits were subjected to preliminary clinical examinations and found to be healthy. The present study was conducted after prior approval from IAEC, in the Department of Veterinary Surgery & Radiology, College of Veterinary Sciences & Animal Husbandry, Narendra Deva University of Agriculture & Technology, Kumarganj, Ayodhya. 224229 (UP). The proposed study is approved by Institutional Animal Ethics Committee reference no. IAEC/CVSc/-ANDUAT/2022/6/1.

Groups

24 adult healthy New Zealand rabbits were randomly allocated into four groups. Theautograftin defected urinary bladders urinary bladder was applied of the rabbits in the experimental groups (Group I). The decellularized EDC cross linked urinary bladder, pericardium and diaphragm of caprine matrix was applied in defected urinary bladders of rabbits(Group II).Rabbits were euthanized at the end of the 30th, 60th and 90th days after surgical experiment.

Anaesthesia

The animals were anesthetized by intramuscular injection of xylazine hydrochloride (6 mg/kg) and ketamine hydrochloride (60 mg/kg) and Complete anaesthesia was obtained within 10 min and the dose was high enough to anaesthetise the rabbits for about half hour (Amarpal *et al.*, 2010) [1].

Surgical procedure

1. After proper anesthesia, rabbits were placed on the operation table in dorso-ventral recumbency.
2. A caudal median laparotomy was performed by incising skin 4-5 cm on the mid ventral/ para-penile aspect of caudal abdomen and the urinary bladder was partially taken out from abdomen.
3. The most nonvascular area was identified in the urinary bladder for creation of defect. The stay sutures was made with 3-0 polypropylene in the directions of 3, 6 (caudal of bladder), 9, 12 (cranial of bladder) o'clock in order to be able to identify the region for creation of the defect where the graft was applied.
4. Partial cystectomy (1cm ×1cm) was performed in all the group of animals and the defect was repaired using autograft and decellularized EDC cross linked urinary bladder, pericardium and diaphragm of caprine matrix scaffolds. The scaffold was sutured to edges of the bladder defect using simple continuous suture technique with a 4-0 vicryl.
5. The suture was applied full thickness into the bladder lumen.
6. The abdominal wall and skin incision was closed routinely.

Post-operative care

1. During the postoperative period, initial five days injection ceftriaxone sodium @ 20mg/kg I/M OD and analgesic meloxicam @ 0.2mg/kg I/MODwas given and surgical dressing was done with povidone-iodine solution.
2. Skin sutures was removed 10 days after surgery.

Clinical observations

Feeding pattern and general behavioral changes in all animals were observed daily during the observation period. Rectal temperature was recorded before operation and on days 1, 2, 3, 5, 7 and 15 after the implantation.

Serum urea nitrogen and creatinine assessment

The serum samples were collected before the operation and on days 30, 60 and 90 after the implantation. The level of serum urea nitrogen and serum creatinine was estimated using biochemistry autoanalyzer (Vitros DT chemistry autoanalyzer).

Biochemical analysis

Blood samples was collected 1 to 2 hr. before surgery (time 0) and at 30, 60 and 90 days after surgery for evaluation of CBC and Serum blood urea nitrogen (BUN) and creatine values.

Statistical analysis

All the data are presented as mean ± standard error of mean. The data were analysed by pair t-test as per Snedecor and Cochran (16). Data were considered statistically significant different if $p<0.05$.

Results

Clinical observations

Animals of different groups started taking feed and water partially within 24 h of operation, but remained slightly dull for 3 days. Post-operatively, animals of all groups passed blood tinged urine for 2 days. During urination all animals assumed a haunched back posture. All animals survived the surgery without any complications; there were no urinary leakage or extravasation of urine. None of the animals died of causes related to immunogenic reactions or clinical rejection.

The rectal temperature increased significantly ($p<0.05$) in all groups upto day 3 post-implantation. The peak value was recorded at day 3. Thereafter, significant ($p<0.05$) decrease was observed from day 5 to day 15 pos-timplantation. At day 15 values were nearly similar to that of the preoperative values.

Hemato-Biochemical observations

White blood cells (WBCs) count

The values of white blood cells (mean±SE) recorded at different time intervals are presented in fig.and table. The levels of WBCs was significantly higher ($p<0.05$) at days 30 post-operatively in all the four groups. The peak value of WBCs was recorded at day 30 in all the animals of different groups. Thereafter, the values of white blood cells showed a declining trend and returned to physiological range at day 90 in all the four groups.

Table 1: Mean ± SE of WBC level of different group of rabbits at different time interval (Normal value 11.5±0.63 X10^3/µl)

Group	Post-operative days		
	30	60	90
I	12.3±0.088	11.9±0.081	11.6±0.070
II	13.2±0.151	12.7±0.143	11.7±0.024
III	13.5±0.116	12.5±0.070	11.5±0.092
IV	12.9±0.077	12.0±0.067	11.4±0.079

Blood urea nitrogen (BUN)

The values of serum urea nitrogen (mean±SE) recorded at

different time intervals are presented in fig. and table. The levels of BUN was slightly higher (p>0.05) at days 30 post-operatively in all the four group of animals. The peak value of BUN was recorded at day 30 in all the animals of different groups. Thereafter, the values of blood urea nitrogen showed a declining trend and returned to physiological range at day 30 and day 90 in all the animals of different groups. The comparison among the different groups showed that BUN values recorded at particular time interval were non significantly (p>0.05) different from each other.

Table 2: Mean ± SE of Blood urea nitrogen level of different group of rabbits at different time interval (Normal value 18.69±0.152 mg/dl)

Group	Post-operative interval (Days)		
	30	60	90
I	18.96±0.692	19.41±0.499	19.17±0.573
II	19.37±0.189	18.71±0.396	19.33±0.735
III	19.21±0.581	18.81±0.849	18.50±0.561
IV	19.49±0.425	19.02±0.743	19.13±0.483

Serum creatinine

The values of serum creatinine (mean±SE) recorded at different time intervals are presented in fig. and table. In the post-operative days there was a non significant (p>0.05) in serum creatinine level and peak value was recorded at day 30. Thereafter, the creatinine values in all the four groups of animals at day 60 and day 90 decline towards base value and did not show any significant difference (p>0.05) from their corresponding pre-operative values. Creatinine is the waste product of creatine metabolism, which is involved in muscle contraction. In excretory dysfunction its blood level increases. It is considered as a better indicator of renal function than urea (Kerr, 2002) [14]. A non significant increase in serum creatinine level in all animals of different groups at day 30, might be the result of impaired bladder function. The stagnation of urine in the bladder possibly interfered with the flow of urine from kidney to bladder. The interference in the free flow of urine from the kidney to bladder combined with possible diffusion of small quantities of urinary constituents across the graft/scaffold and its subsequent absorption into circulation in early stage could be the possible cause of increased serum creatinine levels in all the groups of animals. As the surface area of grafts reduced due to regeneration of uroepithelium and the character of grafts changed due to deposition of fibrin, the serum creatinine levels also decreased and became almost normal by day 60 and 90 in all the groups. Similar findings have also been reported by earlier workers (Gera *et al*., 1980; Reddy, 1991; Ghosh, 1998) [7, 21, 9]. The acellular matrix might have resulted in more diffusion of urinary solutes and subsequent higher serum creatinine for longer duration. Fluctuation of serum creatinine level within normal range has been observed after colocystoplasty in buffalo (Sharma and Khan, 1978) [23] and amniocystoplasty in goats (Reddy, 1991) [21]. Creatinine levels have also been recorded within the reference range (4-18 mg/dl) in all the experimental dogs repaired with homologous bladder acellular matrix graft during the study periods (Probst *et al*., 1997) [20].

Table 3: Mean ± SE of Serum creatinine level of different group of Rabbits at different time interval (Normal value 1.9 ±0.081 mg/dl)

Group	Post-operative interval (Days)		
	30	60	90
I	2.23±0.140	1.65±0.127	2.01±0.098
II	2.63±0.148	1.96±0.054	2.03±0.062
III	2.48±0.191	1.83±0.139	2.06±0.113
IV	2.14±0.147	1.78±0.512	2.00±0.710

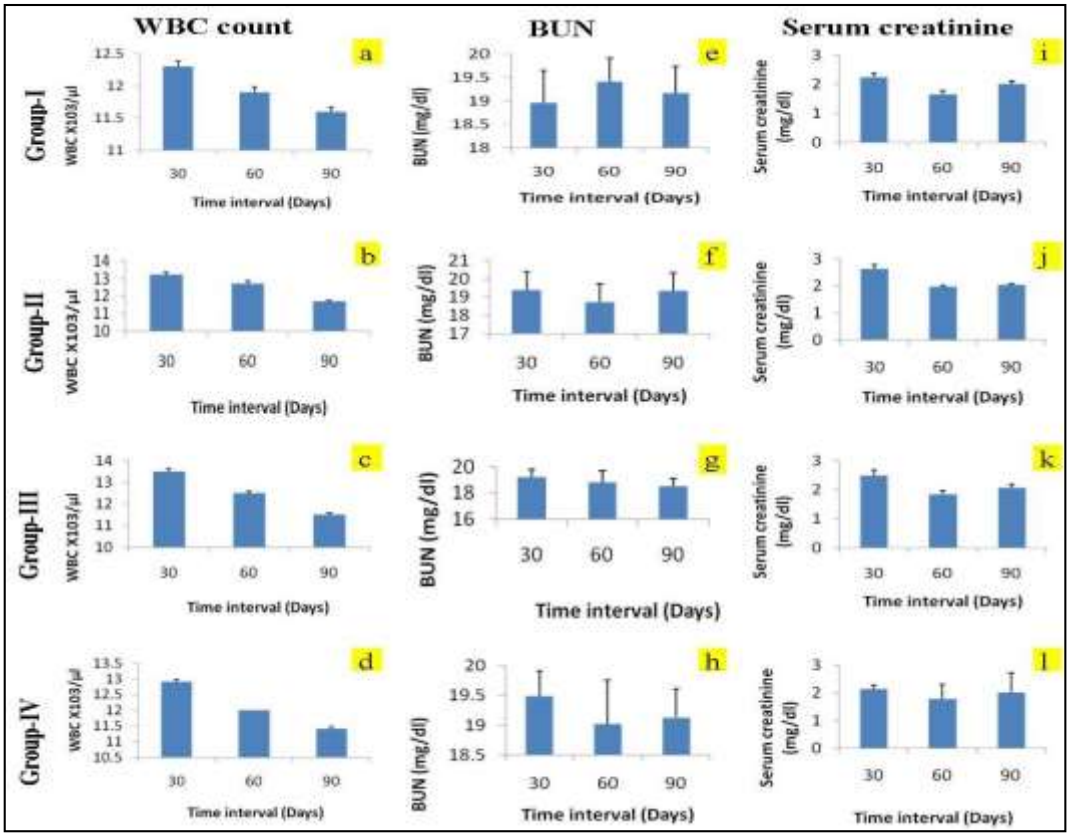

Fig 1a-i: WBC count, Blood Urea Nitrogen and Serum Creatinine concentration in the animals of different group at day 30, 60 and 90

Discussion

In the present study, the rabbits were successfully anesthetized by intramuscular injection of ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (6 mg/kg) without any apparent complication. Complete anaesthesia was obtained within 10 min and the dose was high enough to anaesthetise the rabbits for about half hour (Amarpal *et al.*, 2010) [1]. Other researchers anesthetized the rabbits using half of the dose of xylazine (5 mg/kg) and ketamine (25–30 mg/kg) compared to the present study by intramuscular injection (Kajbafzadeh *et al.*, 2018) [13]. Bladder regeneration surgery was carried out under general anesthesia using intramuscular injection of Ketamine (35 mg/kg) and Xylazine (5 mg/kg) (Sabetkish *et al.*, 2014) [22]. In another study, anesthesia in the rabbits was induced using a combination of ketamine 10 mg/kg, xylazine hydrochloride 3 mg/kg, and acepromazine 0.1 mg/kg intramuscularly. Lidocaine was also used at the projected site of the surgical incision as a topical anesthetic. Before laparotomy, the rabbits were maintained under with 3–4% isoflurane diluted with 100% oxygen prior to surgical incision for autologous adipose tissue collection (Piovesana *et al.*, 2022) [18].

After proper anesthesia, a caudal median laparotomy was performed by incising skin 4-5 cm on the mid ventral/ para-penile aspect of caudal abdomen and the urinary bladder was partially taken out from abdomen. Piovesana *et al*. (2022) [18] performed suprapubic longitudinal laparotomy to access the abdominal cavity for implantation of the graft to repair urinary bladder. Kajbafzadeh *et al.* (2018) [13] exposed the urinary bladder through a midline lower abdominal incision. The most nonvascular area was identified in the urinary bladder for creation of defect. Partial full thickness cystectomy (1cm ×1 cm) was performed in all the animals of different groups and the defects were repaired using autograft and xenogenic EDC cross linked decellularized urinary bladder, pericardium and diaphragm. The autograft and different scaffolds were sutured to edges of the bladder defect using simple continuous suture technique with a 4-0 vicryl. Piovesana *et al.* (2022) [18] excised a rectangular section of 0.8 cm X 1.0 cm from the anterior and apical face of the urinary bladder wall and decellularized vein scaffold was sutured with continuous Vicryl 7.0 sutures at the site. Kajbafzadeh *et al.* (2018) [13] dissected the seromuscular layer until a wide-mouth herniation of bladder mucosa was created. Then an approximately 5 cm^2 acellular scaffold was used to cover the resulting herniation. 6–0 Vicryl sutures were used and care was taken not to penetrate the inner luminal surface. The water-tightness of suture lines was tested by instilling saline into the bladder. Sabetkish *et al.* (2014) [22] augmented the bladder with acellular patch using 6-0 Vicryl sutures in a water tight fashion. The abdominal wall was sutured in standard manner. Each rabbit was administered ceftriaxone sodium (20mg/kg, IM) intramuscularly for five days and analgesic meloxicam (0.2mg/kg, IM) were given for the first 3 postoperative days. Piovesana *et al.* (2022) [18] administered enrofloxacin (10 mg/kg) intramuscularly for 7 d with additional analgesia with tramadol hydrochloride (5 mg/kg) if needed. Enrofloxacin was administered for all rabbits postoperatively (Kajbafzadeh *et al.*, 2018) [13]. The animals were housed in separate cages and monitored daily up to the end of study.

The rectal temperature of all the animals of different groups was within normal range except in two animals of group II in which hypothermia developed two day after surgery. These animals collapsed on day 5 possibly due to leakage of urine from the urinary bladder in to the peritoneal cavity through the implanted decellularized urinary bladder scaffold.

Researchers interested in the medical conditions of animals in clinical practice can benefit from knowing the reference values of rabbits' haematological and biochemical parameters (Lepitzki and Woolf, 1991) [15]. Haematological changes often reveal the existence of stressors, diverse visceral organ infections, necrosis, and inflammation (Jurcik *et al*., 2007) [12]. According to reports, a rise in the WBC count in rabbits seldom reflects an illness; instead, it often changes as a result of varied stressors and blood collecting techniques (Silva *et al*., 2005) [5]. Based on research, rabbits housed alone had higher WBC counts than those fed in groups (Fuentes and Newgren, 2008) [6]. In this study, the values of WBC count in rabbits is presented which were in the range of normal values as reported in previous studies (Hewitt *et al*., 1989) [10]. In the groups II, III and IV, the increase in WBC count values were higher, though within normal range, were persisted for longer period than group I which might be attributed to the histological makeup of the acellular graft. The acellular matrix might have resulted in more diffusion of urinary solutes and subsequent higher WBC level for longer duration.

The biochemical alternations following rupture of urinary bladder are due to two fold effects, i.e. progressive accumulation of urine and distension of urinary bladder prior to its rupture and subsequent accumulation of large volume of urine in the abdominal cavity. Since peritoneal surface is responsive to some of the constituents of urine, homeostatic changes and acid-base disturbance occur due to absorption and/or prolonged accumulation of urine in the peritoneal cavity. Adequate information is available in this regard in different ruminants (Brobst *et al*., 1989) [3]. But, the present experimental investigation does not fall under this clinical situation. In this study, where cystoplasties were evaluated, a piece of bladder was removed and autograft/decellularized scaffolds were grafted concurrently under monitored situation. Alterations in the relevant biochemical parameters have been reported in cattle (Gera *et al*., 1973) [8] and goats (Ghosh, 1998) [9]. Considering the physiological response, estimation of serum urea nitrogen and serum creatinine in this study were undertaken to elucidate their probable association after grafting. The objective of these parameters which are related to the urological functions served not only to support the clinical findings, but also to suggest therapy with a precision that is not possible by clinical observation alone. This contention guided to select the biochemical parameters which have been evaluated in the present study. In subtotal cystoplasty using caecal pedical graft in dogs, increase in plasma urea concentration was observed to a considerable extent up to fifth week (Prasad *et al*., 1977) [19], which was due to the free exchange of water, urea and electrolytes across the intestinal mucosa. But in this study, no viable segments of biological materials were used which had independent circulatory profile other than the bladder mucosa. However, increased BUN in buffalo after accomplishing cystoplasty with preserved bladder (Gera *et al*., 1980) [7] and in goats following cystoplasty with PTFE as well as caecal graft (Shivaprakash, 1990) [24] and amnion (Reddy, 1991) [21] and allogenic acellular bladder (Ghosh, 1998) [9] has been reported. The probable reason of elevated blood urea nitrogen following cystoplasty might be through diffusion of urine

from anastomotic site and from grafts itself in all the four groups. The reconstructed bladder might have resulted to incomplete emptying of urinary bladder and initial diffusion of urine along the suture line and its subsequent absorption. This diffusion possibly was more in immediate post-operative day which resulted in peak blood urea nitrogen on day 30. The higher blood urea nitrogen content in all the animals of different groups for first 30 days were probably due to the divergent nature of the grafts/scaffolds. After bladder repair, serum urea nitrogen steadily dropped and reached preoperative levels by day 30 (Dewangan *et al*., 2013) [26]. The acellular matrix by virtue of its more intracellular and intercellular space might have resulted in more diffusion of urine into peritoneal cavity and subsequently increased the blood urea nitrogen level. It was further supported by the fact that, as the urinary bladder regained the normal function with the regeneration of host tissue in the grafted areas, the blood urea nitrogen values also came down to the base line level in the animals of all the groups irrespective of grafts used. These findings are in agreement with the observations of earlier workers (Shivaprkash, 1990; Ghosh, 1998) [24, 9].

The values of serum creatinine in the post-operative days there was a non significant (p>0.05) in serum creatinine level and peak value was recorded at day 30. Thereafter, the creatinine values in all the four groups of animals at day 60 and day 90 decline towards base value and did not show any significant difference (p>0.05) from their corresponding pre-operative values. Creatinine is the waste product of creatine metabolism, which is involved in muscle contraction. In excretory dysfunction its blood level increases. It is considered as a better indicator of renal function than urea (Kerr, 2002) [14]. A non significant increase in serum creatinine level in all animals of different groups at day 30, might be the result of impaired bladder function. The stagnation of urine in the bladder possibly interfered with the flow of urine from kidney to bladder. The interference in the free flow of urine from the kidney to bladder combined with possible diffusion of small quantities of urinary constituents across the graft/scaffold and its subsequent absorption into circulation in early stage could be the possible cause of increased serum creatinine levels in all the groups of animals. As the surface area of grafts reduced due to regeneration of uroepithelium and the character of grafts changed due to deposition of fibrin, the serum creatinine levels also decreased and became almost normal by day 60 and 90 in all the groups. Similar findings have also been reported by earlier workers (Ghosh, 1998) [9]. The acellular matrix might have resulted in more diffusion of urinary solutes and subsequent higher serum creatinine for longer duration. Fluctuation of serum creatinine level within normal range has been observed after colocystoplasty in buffalo (Sharma and Khan, 1978) [23] and amniocystoplasty in goats (Reddy, 1991) [21]. Creatinine levels have also been recorded within the reference range (4-18 mg/dl) in all the experimental dogs repaired with homologous bladder acellular matrix graft during the study periods (Probst *et al*., 1997) [20].

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