

Original Article

Decellularization of Caprine Urinary Bladder Using *Sapindus Mukorossi* Fruit Pericarp Extract

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In the present study herbal detergent has been used for decellularization of caprine urinary bladder matrix to develop scaffolds for use in regenerative medicine. Urinary bladder was delaminated and decellularized by hypertonic solution and 5% aqueous extract of *Sapindus mukorossi* fruit pericarp, respectively. Complete delamination was observed at 9±1h interval. The delaminated urinary bladder tissues were completely decellularized at 72 h as evidenced by histology, DAPI staining, scanning electron microscopy and estimation of DNA content. The herbal decellularization system removed ~94% of tissue's DNA, preserving ECM's microarchitecture. The non-enzymatic and collagenase (20U/ml) digestion test proved that the glutaraldehyde treatment significantly improve the biostability of these scaffolds. The free protein and free amino acid group concentration of uncross-linked decellularized matrix was significantly ($P<0.05$) higher than cross-linked tissues at different cross-linking time intervals. The fixation index was highest in tissues cross-linked for 72h. The free hydroxyproline content in the cross-linked acellular scaffolds was nil and moisture contents was significantly lower ($P<0.05$). Crosslinked scaffolds did not show any band pattern in SDS-PAGE gel. It was concluded that soap nut pericarp extract is a viable option for decellularization of caprine urinary bladder and glutaraldehyde treatment enhance its biostability. To the best of our knowledge, this is the first report on decellularization of urinary bladder using herbal detergent.

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Introduction

Reconstruction of tissue defects is a great issue in regenerative medicine. Allografts and xenografts are thus opened up as alternate options but their use is restricted because of shortage of donor tissue, chances of disease transfer and immunologic rejections [1]. Decellularized scaffolds derived from natural tissue have been used extensively as implants to help alleviate the chronic shortage of autologous grafting materials [2]. Tissue decellularization is the process of removal of cellular components to produce acellular extracellular matrix (ECM). Extraction of cellular material and debris from tissue may remove lipid membranes and membrane

associated antigens as well as soluble proteins as a means for reducing the antigenic response to xenograft materials. Decellularized scaffolds act as an inductive template for recellularization [3].

Various biological detergents have been used for decellularization of tissues. However, complete decellularization requires a combination of all three biological, chemical and physical methods [4]. Chemical and/or enzymatic treatment is concerned with the use of chemical reagents like sodium deoxycholate [5-8], hypertonic solutions [3], sodium dodecyl sulphate (SDS) [7], Triton X-100 and DNase [9]. However, these detergents cause damage to collagenous and non-collagenous proteins, GAGs and growth factors. The use of chemical and enzymatic agents raises some concerns due to the potential for the presence of residual cytotoxic agents in the decellularized ECM [10].

The fruit of *Sapindus mukorossi*, a member of the family

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Figure 1: Dried fruits, pericarp and seeds of *Sapindus mukorossi*

Sapindaceae, is valued for the saponins (10.1%) present in the pericarp and forms up to 56.5% of the drupe. Soap nut pericarp contains triterpenoidal saponins namely oleanane, dammarane and tircullane [11]. These saponins are secondary metabolites used as herbal detergent due to its amphiphilic nature with the presence of a lipid-soluble aglycone and water-soluble chains in their structure [12]. This herbal detergent has not yet been used for decellularization of tissues. It also has anti-inflammatory, antimicrobial properties antifungal, anti-parasitic and antiviral [13,14]. *Sapindus mukorossi* fruit pericarp extract have been used for decellularization of Caprine gall bladder, aorta and esophagus [15-17].

The urinary bladder is a complex organ, whose main functions are storage of urine under low and stable pressure and micturition. The bladder could be damaged or lost via wide range of injuries, necessitating subsequent replacement or repair. Currently applied surgical procedure utilizing bowel segments are associated with numerous complications, which involve mucus production, chronic bacteriuria, stone formation, ruptures, leakages and fibrosis [18]. So, there is still a need for an 'off the shelf' scaffolds suitable for urinary bladder tissue repair.

Several methods of decellularization have been described for tissue engineering of bladder but there is not a single report on decellularization of urinary bladder matrix using herbal detergent. Therefore, the present study was designed to evaluate the efficacy of 5% aqueous extract of the fruit pericarp of a plant, *Sapindus mukorossi* for decellularization of urinary bladder



Figure 2: Macroscopic appearance of caprine urinary bladder

matrix of caprine origin and to assess its *in vitro* biocompatibility after cross-linking with glutaraldehyde.

Materials and Methods

Preparation of soap nut pericarp extract

Soap nut pericarp extract was prepared as per method described by Ghagi and coworkers [19]. Briefly, commercially available well dried ritha fruits (*Sapindus mukorossi*) were purchased from local market. Pericarp was separated from seed (figure 1) and 5 gm of finely powdered pericarp was soaked overnight in 100 ml phosphate buffer saline (PBS) at 23°C. This mixture was further vortexed using magnetic stirrer for 2 h at room temperature and then filtered using stainless steel sieve. The ritha concentration in this solution was determined on the basis of the ratio of weight of ritha pericarp to that of PBS volume. Thus the concentration of ritha in the above mentioned solution becomes 5 wt %. This solution was further subjected to centrifugation at 5,000 rpm for 20 min and at room temperature to get suspended free supernatant solution which was used for further studies.

Procurement and cleaning of caprine urinary bladder

Caprine urinary bladder (figure 2) was procured aseptically from the local abattoir and stored in chilled sterile phosphate buffered saline (PBS) containing (0.1% amikacin) and proteolytic inhibitor (0.25% EDTA). The samples were washed thoroughly with sterile phosphate buffered saline (PBS, pH 7.4) to remove adherent blood and debris. The maximum time period between retrieval and the initiation of protocol was less than 4 h.

Delamination and decellularization

After initial washing, the native urinary bladder was cut into 1x1cm² pieces and transferred into a sterilized plastic container having hypertonic solution and continuously agitated up to 12 h. After 6 h, the samples were collected at 1 h interval for confirmation of complete delamination. The delaminated tissues were transferred to 100 mL of 5% extract of soap nut pericarp and subsequently agitated on magnetic stirrer for 72 h at room temperature. Tissue samples were collected at 6, 12, 24, 48 and 72 h time intervals for confirmation of decellularization by histological examination, DAPI staining, scanning electron microscopy (SEM) and DNA estimation. All the samples were stored in PBS solution containing 0.1% amikacin at - 80°C until use.

Verification of cell removal

Histological observations

For histological observations, the tissue samples were fixed in phosphate buffered 10% formalin saline, dehydrated in ethanol, cleared in xylene and embedded in paraffin to get 5 micron thick paraffin sections. The sections were stained with hematoxylin and eosin and evaluated microscopically by using histological scoring system [5]. The histological parameters were graded as follows: cellular contents: normal cellularity (+++), moderate number of cells (++), mild number of cells (+), no cellular material (-); debris: more (+++), moderate (++), mild (+), no debris (-); collagen fibers arrangement: compact (+++), mildly loose (++), moderately loose (+), heavily loose (-); porosity: highly porous (+++), moderate (++) and mild (+).

DAPI staining: (4, 6-diamidino-2-phenylindole 2HCl)

Native and decellularized urinary bladder tissues were fixed in 10% formalin. These tissues were processed and sectioned in standard manner. The sectioned tissue was loaded over the amino propyl

tri-ethoxy silane (APTES) coated slides. The sections were dewaxed in xylene (2 x 5 min), rehydrated in ethanol series (absolute, 95% for 5 min, 70%, 30% ethanol, dH₂O for 3 min). The slides were washed with 0.2%TBST (Tris buffered saline Tween 20) for 2-3 times. The slides were dried on paper towel, DAPI staining solution was applied on each slides (~200 µl), incubated for 15 min in dark at room temperature. The slides were washed with 0.2%TBST (Tris buffered saline Tween 20) for 2-3 times, 3-5 minutes each to remove the unbind DAPI. Mount cover slips with Gel Mount. Nuclei emit cold blue fluorescence under fluorescent microscope.

Scanning Electron Microscopy (SEM)

The SEM examination of the native and decellularised urinary bladder samples was performed. Scanning electron microscope (SEM) model Jeol JSM-840 was used for ultrastructure observations. Briefly, the bladder samples were cut into small pieces and fixed into freshly prepared karnovsky fixative at 4°C. After fixation for 3-4 days tissues were washed three times with phosphate buffer of pH 7.2. Specimens were dehydrated using serial gradient of alcohol in water. Finally tissues were washed three times in absolute alcohol and treated with Hexamethyldisilazane (HMDS). Specimens were mounted on aluminum stubs using adhesive carbon tape and sputtered with gold ions at 1.2 kV (7 mA current) for 5 min. Finally specimens were observed under SEM at 5 kV using different magnifications as needed for the desired observations.

DNA quantification

To verify the extent of decellularization, tissue specimens for DNA quantification were taken from native and decellularized bladder tissue (25 mg each) and placed on dry filter paper to remove excess fluid. Each sample was homogenized separately with a mortar and pestle. Total genomic DNA was isolated from native and decellularized scaffolds using the DNASure® Tissue mini kit (Genetix Biotech Asia Pvt. Ltd.) following the manufacturer's instructions. The total amount of DNA was quantified using spectrophotometry (NanoDrop ND-1000, NanoDrop Technologies).

Cross-linking of the acellular urinary bladder matrix

Decellularized bladder was cross-linked with glutaraldehyde (GA) (0.6% in PBS) at room temperature. The amount of solution used to cross-link each sample was 20 ml and changed at 24 h time interval. Tissues of each study group were kept for 12, 24, 48 and 72 h in chemicals for cross-linking under constant agitation on orbital shaker. The cross-linking was done at room temperature. Decellularized tissues preserved in normal saline were used as control.

In vitro evaluation of cross-linked acellular caprine urinary bladder matrix

In vitro biocompatibility determination was done on the basis of the following parameters:

Gross observations: Gross observations of tissue after cross-linking include change in colour, consistency, swelling and stiffness.

In vitro non-enzymatic degradation: The acellular matrix was subjected to *in vitro* non-enzymatic degradation tests. Preweighed dry specimens were immersed for 0, 1, 3, 5 and 7 days at 37°C in isotonic saline solution containing 0.1% sodium azide (NaN₃) [20]. The values of weight loss were expressed in percentage.

In vitro enzymatic degradation: The cross-linked urinary bladder tissues were equilibrated overnight in PBS with 0.2 mg/ml sodium

azide (NaN₃) as preservative. The samples were then removed from the solution, excess moisture was blotted from the surface and the initial mass was recorded. The samples were transferred to 2 ml micro centrifuge tube and 1.75 ml of 20 U/ml Collagenase Type-I from *Clostridium histolyticum* in PBS with 0.2mg/ml sodium azide was added to each tube and incubated for 12, 24, 48, and 72 h intervals at 37°C [21]. The tissues were blotted and the mass was determined. Weight loss of biomaterial was then calculated that of the original tissue. The values of weight loss were expressed in percentage.

Free amino group content determination: Ninhydrin assay was used to determine the free amino group contents of each test sample after cross-linking [22]. The sample was dried for 24 h and weighed. Subsequently the dried tissue was heated with ninhydrin solution on water bath for 20 minutes. After heating with ninhydrin, the optical absorbance of the solution was recorded with spectrophotometer at a wavelength of 570 nm. Standard curve was prepared using glycine (20 µg, 40 µg, 60 µg, 80 µg, 100 µg and 150 µg/ml). The absorbance was recorded and linear standard graph was obtained.

Fixation Index determination: The fixation index was determined by ninhydrin assay [22].

$$\text{Fixation Index (\%)} = \frac{(\text{NHN reactive amine})_{\text{fresh}} - (\text{NHN reactive amine})_{\text{fixed}}}{(\text{NHN reactive amine})_{\text{fresh}}} \times 100$$

Estimation of free hydroxyproline content: The hydroxylproline content of test samples was estimated using hydroxyproline stock solution as a standard [23]. Briefly, take 100 µl of each test sample and 100 µl of 2.5 µg, 5 µg, 10 µg, 15 µg, and 20 µg of hydroxylproline standard samples. In each test tube 100 µl of 0.05M copper sulphate, followed by 100 µl of 2.5N sodium hydroxide was added. The test tubes were placed in a water bath at 40°C for 3-5 minutes. Add 100 µl, 6% hydrogen peroxide and left in the water bath for 10 min. The test tubes were cooled with tap water and 400 µl of 3N H₂SO₄ and 200 µl of 5% p-dimethyl-aminobenzaldehyde solution were added. Caps were placed on the test tubes and were kept in a water bath at 70°C for 16min. The solutions were then cooled and absorbance was read at wavelength of 550nm. Sample concentrations were determined from the standard addition curve.

Moisture content analysis: The wet tissue was sandwiched between the two paper towels and a 40 g weight was applied on top for 10 sec. The weight of the tissue was recorded as wet tissue weight. Subsequently, the tissue was dried for 24 h and weighed again (dry tissue weight). Finally, the moisture contents of the test tissue were calculated using the following formula [22].

$$\text{Moisture content (\%)} = \frac{\text{Weight(wet tissue)} - \text{Weight(dry tissue)}}{\text{Weight(wet tissue)}} \times 100$$

Moisture content was determined in triplicate for each specimen as percentage of initial weight loss during drying.

Molecular weight analysis: Molecular weight analysis of the native, acellular and cross-linked (12h, 24h, 48h and 72h) acellular urinary bladder samples was performed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In brief, 100mg of each test sample was triturated with 10% SDS (1mL) and supernatant was obtained after centrifugation at 10,000 rpm for 10 minutes. The supernatant were mixed with an equal volume of 1x non-reducing sample buffer and using 10% polyacrylamide gel under non-reducing [24]. After electrophoresis the gels were removed and stained with 0.5% Coomassie brilliant blue R-250 dye in 30% methanol and 10% acetic acid for three hours. After staining, it was unstained with three changes of 30% methanol and 10% acetic acid

for 15, 30 and 60 minutes, respectively for each change. Known molecular weight marker was used to calibrate the gel. The values of different molecular weight protein bands were expressed in kDa.

Statistical analysis

SPSS software version 17.0 (SPSS, Inc., Chicago, IL) was used for data analysis. One way ANOVA (Analysis of variance) was used to compare the mean values at different intervals with their base values. Independent “t” test was used to compare the mean values between groups at different intervals.

Results

Delamination

Separation of the tunica serosa, tunica muscularis externa, tunica submucosa, and most of the mucosa was started at 6 to 8 h time interval mechanically in broken pieces at places. Complete delamination of the urinary bladder was observed at 9 ± 1 h interval. Immersion in hypertonic solution resulted in removal of urothelium, however, cells were evident in the lamina propria

Verification of decellularization

Macroscopic observations

At 12 h the urinary bladder tissue treated with 5% solution of soap nut extract were slightly soft in consistency and slightly yellowish than native tissue. At 24 h the samples were slightly swollen. Thereafter, the consistency of the sample was more or less similar to the 24 h samples and slightly changed up to 72 h interval. The scaffold was white in color (figure 3).

Microscopic observations

The microscopic observations of the native and decellularized urinary bladder scaffold are presented in the table 1 and figure 4a and b. At 6 h and 12 h interval, scaffolds showed 80 to 85% decrease in the cellular contents. The collagen fibres were mildly thick and loose. At 24h the scaffolds showed 90 to 95% decrease in the cellular contents and at 48h the scaffolds were decellularized but debris were present at places. The collagen fibres were mildly thick and mildly loose in architecture. At 72 h the scaffolds were completely acellular with mildly thick and mildly loose collagen fibres and no debris was observed between the collagen fibres.

DAPI staining

DAPI staining of native urinary bladder (figure 4c) showed cold



Figure 3: Macroscopic appearance of decellularized urinary bladder scaffold (72h)

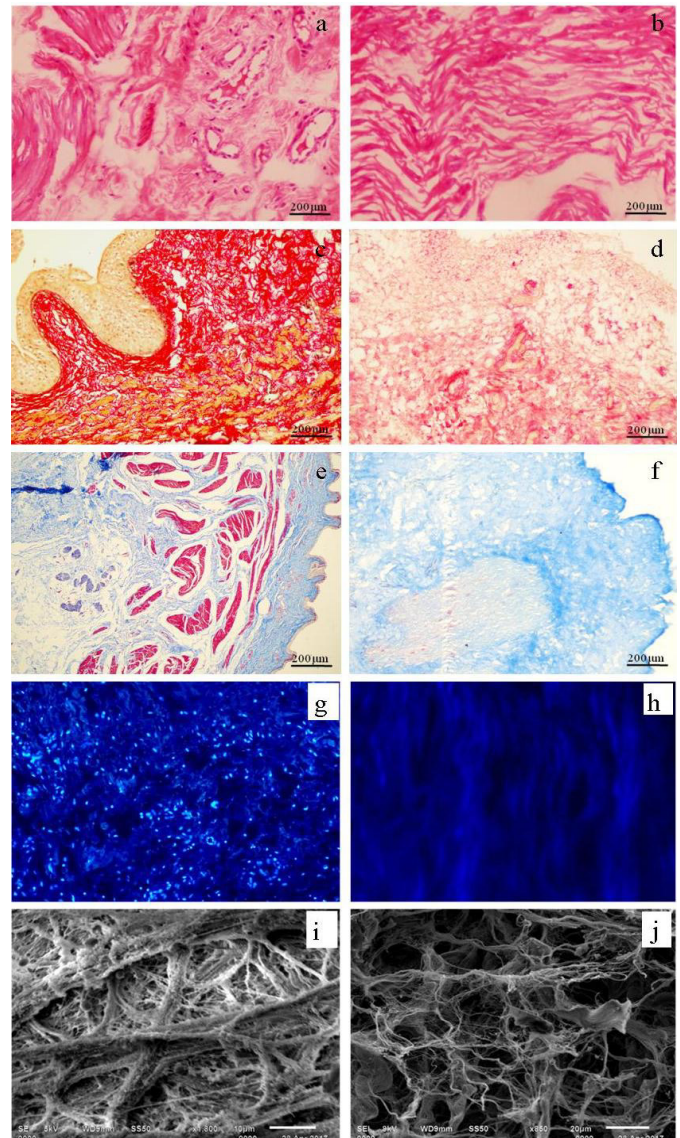


Figure 4: a. Micrograph of caprine native (normal) urinary bladder (H&E stain, 100x). b. Micrograph of caprine urinary bladder after processing (72 h) in 5% extract of soapnut pericarp showing acellular matrix (H&E stain, 100x) c. Picrosirius red stained (PSR) native (normal) urinary bladder matrix showing collagen fibers (red) on yellow background d. PSR stained decellularized urinary bladder matrix (72h) e. Masson's trichrome stained (MTS) native (normal) urinary bladder matrix showing collagen fibers (blue) f. MTS stained decellularized urinary bladder matrix (72h) g. 4',6-Diamidino-2-phenylindole (DAPI) staining of a native (normal) urinary bladder matrix showing cold blue fluorescent Nuclei. h. DAPI staining of a decellularized urinary bladder matrix (72h). i. Scanning electron microscopic image of a normal urinary bladder matrix showing foam configuration with fibroplasia over the collagen fibers. Scale bar 10 μm. j. Scanning electron microscopic image of decellularized urinary bladder matrix showing randomly oriented collagen fibres with large interconnected pores and cells were absent (72h). Scale bar 20 μm.

Table 1: Microscopic observations of caprine urinary bladder processed by 5% extract of soap nut pericarp at different time intervals

Parameters	Time interval					
	Native	6 h	12 h	24 h	48 h	72 h
Cellular contents	+++	++	++	+	-	-
Cellular debris	-	++	+	+	-	-
Collagen fibers	+++	+++	+++	++	+	+
Arrangement	+	+	+	++	++	+++

blue fluorescence which depicts the nuclear DNA. The soap nut extract processed samples of urinary bladder at 24 h and 48 h interval showed few DNA remnants at places. However, the samples collected at 72 h showed complete removal of nuclear components (figure 4d).

Scanning electron microscopy

Native urinary bladder resembled a foam configuration with fibroplasia over the collagen fibers (figure 4e). However, decellularized urinary bladder matrix showed randomly oriented collagen fibres with large interconnected pores and cells were absent (figure 4f).

DNA quantification

Tissue specimens for DNA quantification were taken from native and decellularized urinary bladder. Quantitative evaluation of DNA in the decellularized tissues by spectrophotometer demonstrated a significant reduction of DNA content. Quantity of dsDNA in native and decellularized urinary bladder was 55.4 ± 2.3 ng per mg dry weight and 2.7 ± 0.6 ng per mg dry weight, respectively (figure 5).

Cross-linking and in vitro determination of biocompatibility of acellular urinary bladder matrix of caprine origin

Cross-linking was done by placing the $1 \times 1 \text{ cm}^2$ tissue samples in 30 ml solution of glutaraldehyde for 12, 24, 48 and 72 h duration at room temperature. Glutaraldehyde cross-linked bladder matrix was

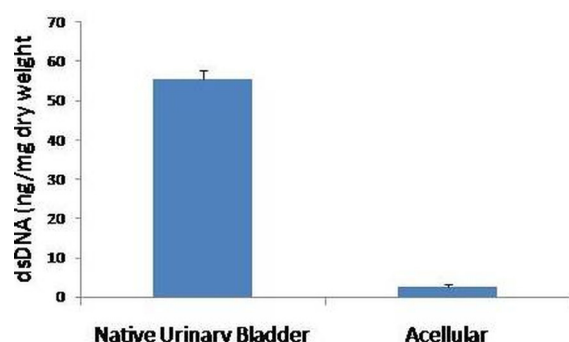


Figure 5: DNA quantification values (ng per mg dry weight) in the native and GA cross-linked acellular urinary bladder matrix. Data represent mean $\pm$ SE (n = 6), *Significant difference, $P < 0.01$

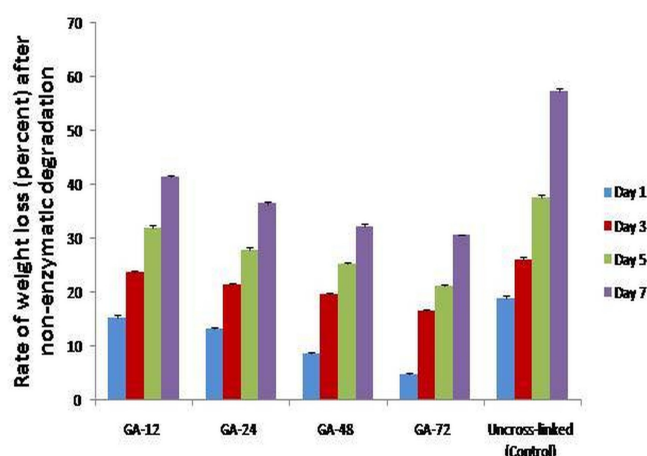


Figure 6: Rate of weight loss (percent) after non-enzymatic degradation (in isotonic saline solution containing 1% sodium azide) of acellular and GA cross-linked acellular urinary bladder matrix. Data represent mean $\pm$ SE (n = 6), *Significant difference, $P < 0.05$

light yellowish; slightly shrunken, hard and stiffer in consistency as compared to uncrosslinked biomaterials.

In vitro non-enzymatic degradation

In vitro non-enzymatic biodegradation of the decellularized urinary bladder scaffolds before and after GA treatment is presented in figure 6. The weight loss increased with increase in digestion time interval in acellular as well as cross-linked tissues. Among all the cross-linked tissues, weight loss was minimal in tissues cross-linked with GA for 72 h.

In vitro enzymatic degradation

In vitro enzymatic biodegradation of the urinary bladder scaffolds before and after GA treatment is presented in figure 7. As the time interval increased, the weight loss significantly ($P < 0.05$) increased in the acellular tissue as compared to GA treated tissues.

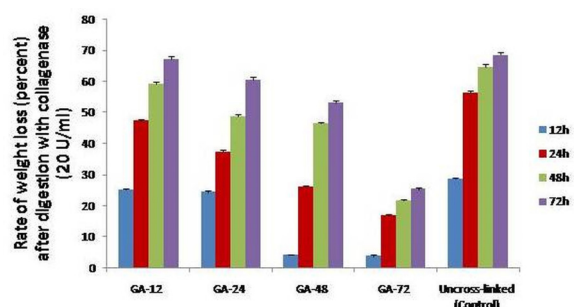


Figure 7: Rate of weight loss (percent) after collagenase (20 U/ml) digestion of acellular and GA cross-linked acellular urinary bladder matrix. Data represent mean $\pm$ SE (n = 6), *Significant difference, $P < 0.05$.

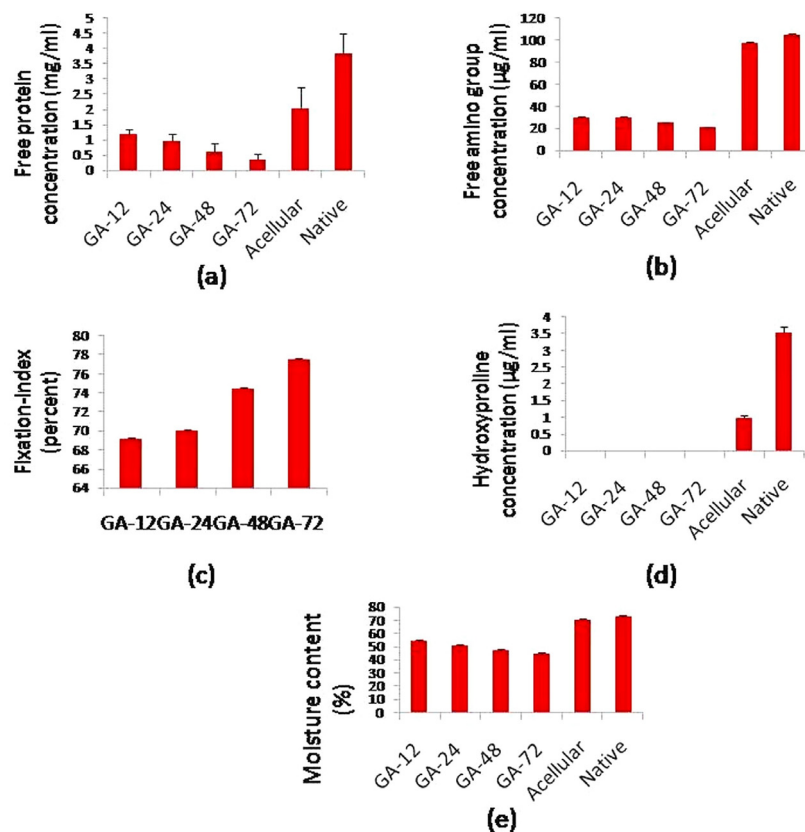


Figure 8: Mean $\pm$ SE of a. free protein concentration (mg/ml) b. free amino group concentration (μ g/ml) c. fixation-index (percent) d. hydroxyproline concentration (μ g/ml) e. moisture content (percent), Data represent mean $\pm$ SE (n = 6), *Significant difference, $P < 0.05$.

Free protein contents

Free protein concentration (mg/ml) of uncross-linked and cross-linked acellular urinary bladder matrices are presented in figure 8a. The free protein concentration in decellularized bladder was significantly ($P < 0.05$) higher than cross-linked tissues at different cross-linking time intervals. Among cross-linked tissues, free protein concentration was lowest in tissues cross-linked with GA for 72 h.

Free amino group contents determination

Figure 8b shows the free amino group concentrations of uncross-linked and cross-linked acellular bladder scaffold. The free amino acid group concentrations of uncross-linked acellular urinary bladder was significantly ($P < 0.05$) higher when compared to cross-linked tissues. In cross-linked tissues, free amino group concentration at 48 h and 72 h cross-linking intervals was significantly ($P < 0.05$) lower as compared to 12 h and 24 h cross-linking intervals.

Fixation index determination

The fixation index (percent) of cross-linked bladder extracellular matrix is presented in figure 8c. In cross-linked tissues, fixation index was highest at 72 h.

Free hydroxyproline content

The free hydroxyproline content in cross-linked, uncross-linked acellular urinary bladder and native urinary bladder are presented in

figure 8d. The free hydroxyproline content in native and acellular bladder were significantly ($P < 0.05$) higher when compared to cross-linked tissues.

Moisture content analysis

Moisture content (percentage) of uncross-linked and cross-linked extracellular bladder matrices is presented in figure 8e. Moisture contents were significantly ($P < 0.05$) higher in acellular tissues when compared to cross-linked tissues.

Molecular weight analysis

The protein bands of native, uncross-linked acellular and cross-linked acellular urinary bladder matrix are visualized in figure 9. The collagen pattern is represented in the native urinary bladder (Lane 2). In the SDS resolving gel, the collagen bands showed molecular weight of about 29, 33, 43, 54, 71, 91 and 124 kDa. Native collagen molecules remained in the stacking gel. After decellularization process, the soluble protein decreased as revealed in SDS-PAGE of acellular urinary bladder matrix (Lane 3-6). The protein band pattern of collagen in acellular urinary bladder matrix in SDS-PAGE did not show any high protein band. The GA treated acellular urinary bladder matrix did not show any higher protein band pattern in SDS-PAGE gel. The GA cross-linked collagen resulted in the formation of large covalently cross-linked complex. This complex did not disassociate by chemical treatment with sample buffer and was too large to enter the stacking gel. Therefore,

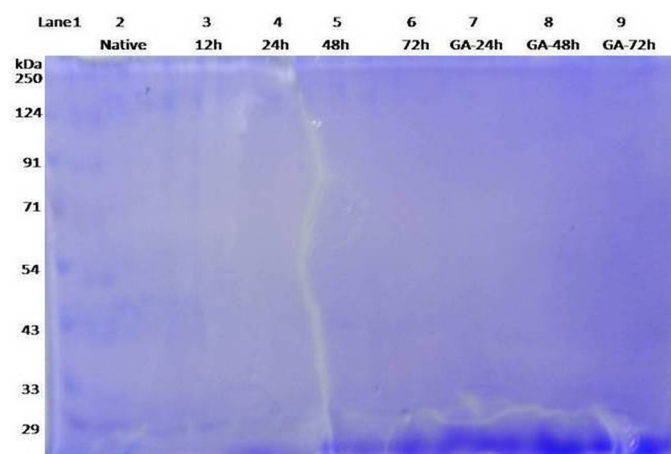


Figure 9: SDS-PAGE of Caprine urinary bladder. Lane 1: ladder, Lane 2: Native urinary bladder, Lane 3: Urinary bladder matrix (UBM)-12h, Lane 4: Urinary bladder matrix-24h, Lane 5: Urinary bladder matrix-48h, Lane 6: Decellularised urinary bladder matrix-72h (DUBM), Lane 7: GA-24h treated DUBM, Lane 8: GA-48h treated DUBM, Lane 9: GA-72h treated DUBM

all the GA treated acellular urinary bladder matrix did not show any band pattern in SDS-PAGE gel (Lane 7-9).

Discussion

The bladder could be damaged or lost via wide range of injuries, necessitating subsequent replacement or repair. Bladder tissue cannot be easily replaced due to its elasticity and urothelial permeability [18]. In the present study, caprine urinary bladder was decellularized by herbal detergent for use in regenerative surgery. The muscle and mucosal layers of urinary bladder were completely delaminated by hypertonic solution. However, hypertonic solution was ineffective in decellularizing stromal tissues [25].

With an effort to minimize the use of toxic chemicals/reagents/enzymes, the delaminated tissues were decellularized using 5% extract of soap nut pericarp under continuous agitation on magnetic stirrer for 72 h at room temperature. The samples were processed without the use of antibiotic or antifungal agents because the soap nut includes the antimicrobial and fungicidal activities [26]. There is about 10% saponin, a natural non-ionic surfactant, in fruit pulp of *Sapindus mukorossi*. Saponin has a good emulsifying, separating and dispersing capability. It is also a good foam stabilizer with a great cleaning capacity [27]. Decellularization efficacy was initially determined by macroscopic visualization. The tissue became white due to progressive cell removal.

Decellularization efficiency of different chemicals used for this purpose can be evaluated by histology (H&E staining), DAPI (4, 6-diamidino-2-phenylindole 2HCl) fluorescent staining, scanning electron microscopy and DNA quantification of the decellularized scaffold. Hematoxylin-eosin staining of decellularized samples showed complete decellularization of the native urinary bladder. The cell extraction along with removal of residual cellular components was effectively achieved without significant disturbances in extracellular matrix (ECM) morphology. During the process of tissue

decellularization, preservation of the native ultrastructure and composition of the ECM is highly desirable [28]. Sodium deoxycholate is very effective for removing cellular remnants, but tends to cause greater disruption to the native tissue architecture when compared to sodium dodecyl sulphate [4]. Bladder acellular scaffolds were prepared using rigorous technique to remove all cells. Mechanochemical decellularization processes are more effective on removing cellular material and maintaining the main tissue characteristics than chemical approaches alone [29].

DAPI, a fluorescent molecule, binds to the adenine-thymine (AT) clusters in the minor groove of DNA and emit cold blue fluorescence [30]. In the present study, decellularized samples showed nearly complete removal of nuclear components in urinary bladder matrices. It was proposed the three-part criteria for tissue decellularization: (1) no visible nuclei in H&E and DAPI staining, (2) no more than 50 ng DNA/mg dry weight and (3) the remaining DNA should not exceed 200 base pair in length [31].

Scanning electron microscopy of native urinary bladder resembled a foam configuration with fibroplasia over the collagen fibers. Globular bodies were seen within the collagen fiber bundles of the basal lamina. However, decellularized urinary bladder matrix showed randomly oriented fibrillar structure with large interconnected pores and cells were absent. Globular bodies were noted within the collagen fibre bundles of the basal lamina of the native urinary bladder [32].

It is logical that minimizing the amount of foreign DNA present in any type of graft tissue would likely increase the chance of successful implantation due to a reduced immune response [33]. The use of 5% extract of soap nut pericarp resulted in the removal of more than 94% of total DNA. DNA quantification of decellularized scaffolds supported the results of HE staining, DAPI fluorescent staining and scanning electron microscopy. These results are similar to others reported in the literature concerning liver and lungs decellularization [34].

If traces of chemicals remain within the scaffold even after proper washings, then it is likely that they will be toxic to recipient's host cells [4]. Soap nut pericarp extract is an herbal product and it is nontoxic and non immunogenic to host cells. It is used as anti-inflammatory, antifungal, antibacterial, anti-parasitic, antitumor and antiviral agent [14]. By using the aqueous extract of soap nut pericarp, enzyme/chemical detergent treatment and subsequent washing may be an avoidable step while isolating biomaterial-grade scaffolds.

Glutaraldehyde cross-linked acellular urinary bladder was stiff and as the cross-linking time increased the tissue became stiffer and yellowish in colour. The cross-linking produces inter-fibrillar cross-links, which is responsible for stiffness of scaffolds [35]. Upon fixation, glutaraldehyde degrades to glutaric acid, which is light yellowish in colour. Glutaraldehyde cross-linking of collagenous tissues significantly reduced the biodegradation, made them biocompatible and preserved anatomic integrity and flexibility. The cross-linking with glutaraldehyde has also been shown to suppress immunological recognition of the tissue by preventing the display of antigenic determinants and prevent early degradation [36]. The cross-linked acellular urinary bladder showed resistant toward non-enzymatic degradation.

The cross-linked urinary bladder was more resistant to enzymatic degradation when compared to the uncross-linked tissues. This resistance may be due to the inhibition of enzyme substrate interaction via the hidden or altered cleavage sites of the collagen by cross-linking agents [37]. The decrease in free protein content released

from the cross-linked acellular matrix indicated the efficacy of the cross-linking of the biomaterials. Cross-linked bladder scaffold showed significant decrease in the free protein as compared to the uncross-linked acellular and native tissues. Cross-linking of acellular matrices may lead to decrease in free protein content [38]. The cross-linking binds the peptide and form large molecule of protein that was also evident in SDS-PAGE, in which the large molecules were unable to pass through the gel and therefore a particular band remained absent [39]. Cross-linked decellularized bladder matrices showed significantly ($P < 0.05$) lower free amino group concentration compare to acellular matrix (control). Free amino group content in the tissues is inversely proportional to the degree of the cross-linking [40]. Fixation index is used to estimate the percentage of amino groups within the tissue reacted with the cross-linking agent. The fixation index of cross-linked acellular urinary bladder was highest at 72 h indicating less free amino groups in the cross-linked tissue. A higher fixation index implies a lower level of free amino groups left in the fixed tissue [19,41]. In clinical situations, such as tissue repair and wound healing, overproduction and deposition of collagen are required to heal the damaged tissues. 4-Hydroxyproline is a specific amino acid of collagen which is widely used as a factor to estimate the collagen content in biological specimens. The free hydroxyproline content in GA cross-linked tissues was nil. Glutaraldehyde treatment of tissues leads to significant decrease in free hydroxyproline content [42].

Moisture content was significantly higher in uncross-linked acellular urinary bladder when compared to cross-linked tissues. The water binding ability of the acellular bladder could be attributed to their hydrophilicity. The moisture content of the decellularized bladder matrix decreased as the cross-linking degree is increased because of the decrease of hydrophilic group [43]. This might be due to the shrinkage of tissue during fixation which reduces the free volume in tissue and thus expelled some water molecules out of the fixed tissue [7]. Increased cross-linking of collagen causes a decrease of the peptide chain length between cross-links, resulting in decreased swelling of samples when crosslink density increases. Cross-linking reduces the equilibrium moisture per cent of collagenous matrices [44]. Intermolecular cross-linking of collagen *in-vitro* by physical treatment or by chemical agents modified the properties of biomaterials. It reduces the solubility, water absorption and biodegradability of the collagen biomaterials and increases its mechanical properties. Cross-linking reduces the equilibrium moisture per cent of collagenous matrices [41,43]. Cross-linked acellular graft delays the degradation of transplanted tissue, thereby, providing sufficient time for the host body to replace the damaged tissue. The bladder tissues treated with GA showed fewer amounts of low molecular weight proteins, which indicated efficient cross-linking.

Conclusion and Future Prospective

It was concluded that the soap nut pericarp extract, an herbal detergent, is suitable for decellularization of caprine urinary bladder as confirmed by histology (H&E staining), DAPI (4, 6-diamidino-2-phenylindole 2HCl) fluorescent staining, scanning electron microscopy and DNA quantification of the decellularized scaffold. By using the aqueous extract of soap nut pericarp, subsequent washing of the scaffold may be an avoidable step while isolating biomaterial-grade scaffolds. The GA treated acellular matrix grafts were more biostable as compared to uncross-linked scaffolds. Glutaraldehyde cross-linked acellular urinary bladder was found biocompatible *in vitro*. These scaffolds could serve as a xenogenic biomaterial and may be used in bladder reconstruction. Further studies are warranted to identify various growth factors present within this biomaterial.

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