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Optimization of protocols for conversion of porcine urinary bladder into bladder acellular matrix graft (BAMG) using biological detergents and enzymes

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Abstract
The present study was carried out for optimization of protocols for conversion of porcine urinary bladder into bladder acellular matrix graft (BAMG) using different biological detergents and enzymes for tissue engineering. The tissue treated with enzyme and biological detergents at different concentration showed more dissolution and only few remnants of tissue were left and were not appropriate for grafting purpose. Therefore, enzyme treatment was omitted and tissue was directly treated with different concentrations of biological detergents for different time intervals. In the present study, the bladder graft treated with 0.5% SDS for 24 h showed complete acellularity with normal collagen fibres arrangement.

Keywords: Bladder acellular matrix, biological detergent, enzyme, porcine, urinary bladder

Introduction
Bladder replacement or augmentation is required in congenital malformations or following trauma or cancer. Numerous natural and synthetic materials have been used in the experimental and clinical setting for the reconstruction of functionally deficient bladder [6]. Biomaterials which are applied experimentally or clinically may include naturally derived materials such as collagen, small intestine sub mucosa, omentum, skin, preserved bladder and even placenta and synthetic materials such as polyglycolic acid, polylactic acid gelatin sponge, polyvinyl sponge etc. The drawbacks associated with above have created an interest in developing better materials which should be not only non-toxic to the cells, but also elicit bioactive cellular response. Therefore, *in vivo* tissue engineering technologies for bladder reconstruction frequently utilizes naturally derived biodegradable material that is placed into the host cells. Thus, a bladder acellular matrix graft may be an attractive alternative. A variety of methods haven reported for decellularization of bladder but aim should be to preserve the functional homology to replace bladder tissue with full thickness matrix in a like with like fashion [7]. Decellularization is a process which removes cellular antigens but preserves the ultrastructure and composition of extracellular matrix (ECM). Decellularized ECM (DECM) scaffolds have been widely used in various tissue engineering applications. The method of decellularization process affects the mechanical, architectural and bioactive properties of a prepared matrix and its clinical efficacy also. Once when cellular components are removed from xenogenic or allogeneic bladders, the prepared bladder acellular matrix scaffold can be used for repair without any immunological reaction. Bladder acellular matrix (BAM) is a naturally derived biodegradable material, developed for use of bladder substitute [11, 16]. BAM is produced by a detergent and enzymatic extraction process, which removes the cell and soluble matrix components from the extracellular matrix [13]. The BAM is composed of typical collagen and elastin, which is important for urothelial cell support [3]. Such decellularization limits the immunogenicity and potentiates the use of collagenous matrices derived from xenogenic sources [1]. A variety of methods have been reported for decellularization of bladder acellular matrix graft. This process may result in damage to extracellular matrix rendering it not fit for bladder tissue engineering. Therefore, the present study was carried out with objective to optimized protocol for decellularization of porcine urinary bladder tissue in to bladder acellular matrix graft (BAMG) by using biological detergents and enzymes for bladder tissue engineering.

Materials and Methods

(A) Decellularization of porcine urinary bladder into bladder acellular matrix graft (BAMG)

The fresh porcine urinary bladder was collected from the local abattoir in cold sterile phosphate buffer saline (PBS, pH 7.4) solution. The tissues were rinsed with PBS to remove the adhered blood. The maximum time period between the retrieval and the initiation of decellulazation procedure was less than 4 h. The tissue were cut into 2x2 cm^2 pieces and subjected to decellulazation protocol using Trypsin enzyme and four different biological detergents viz., Sodium deoxycolate, Sodium Dodecyl Sulfate, TritonX100 and Tween 20 of different concentration for the preparation of bladder acellular matrix graft (BAMG). Each procedure was performed under aseptic condition.

Decellularization protocol I: (Trypsin + Sodium deoxycolate)
The porcine urinary bladder was cut into six pieces each of 2 x 2 cm^2 in dimension and equally divided into 2 groups (A and B). Group A were treated with 0.1% trypsin solution for 12 h and Group B were treated with 0.5% trypsin solution for 4 h as depicted in the Figure 1. All the trypsin treated tissue samples were thoroughly washed with distilled water for 15-20 min. Tissues of group A were placed in 3 different test tubes each containing 0.5% Sodium deoxycolate and were labeled as 1, 2 and 3 and kept at room temperature (37 ^{0}C) for 12, 24 and 48 h respectively. Tissue samples of group B were treated with 1% Sodium deoxycolate in similar way for three different time intervals as described for the tissues of group A. The tissue samples were thoroughly rinsed thrice after each chemical treatment with PBS for 15-30 min to completely remove of the chemical residues of previous step. The tissue in each solution was continuously agitated at 180 rpm in the orbital shaker to provide better contact of tissue with chemicals.

In decellularization protocol II, III and IV, only biological detergent was changed and rest all procedures viz., concentration, time intervals were similar to protocol I. The biological detergent used for decellularization protocol II, III and IV were Sodium Dodecyl Sulfate, TritonX100 and Tween 20 respectively**.**

The results obtained in above mentioned all the 4 protocols were unsatisfactory. The developed bladder acellular matrix grafts from above protocols were not appropriate for grafting purpose. Therefore, all the 4 protocols were modified in which the trypsin treatment was omitted i.e. Modified decellularization protocols. The tissue samples were directly treated with different biological detergent like Sodium deoxycolate, Sodium Dodecyl Sulfate, TritonX100 and Tween 20 with antibiotic (cefotaxime) for the preparation of bladder acellular matrix graft and the concentration of biological detergents, the treatment time and temperature were same as described in original protocols. The tissue in each solution was continuously agitated at 180 rpm at 37 ^{0}C in the orbital shaker to provide better contact of tissue with chemicals in each protocol.

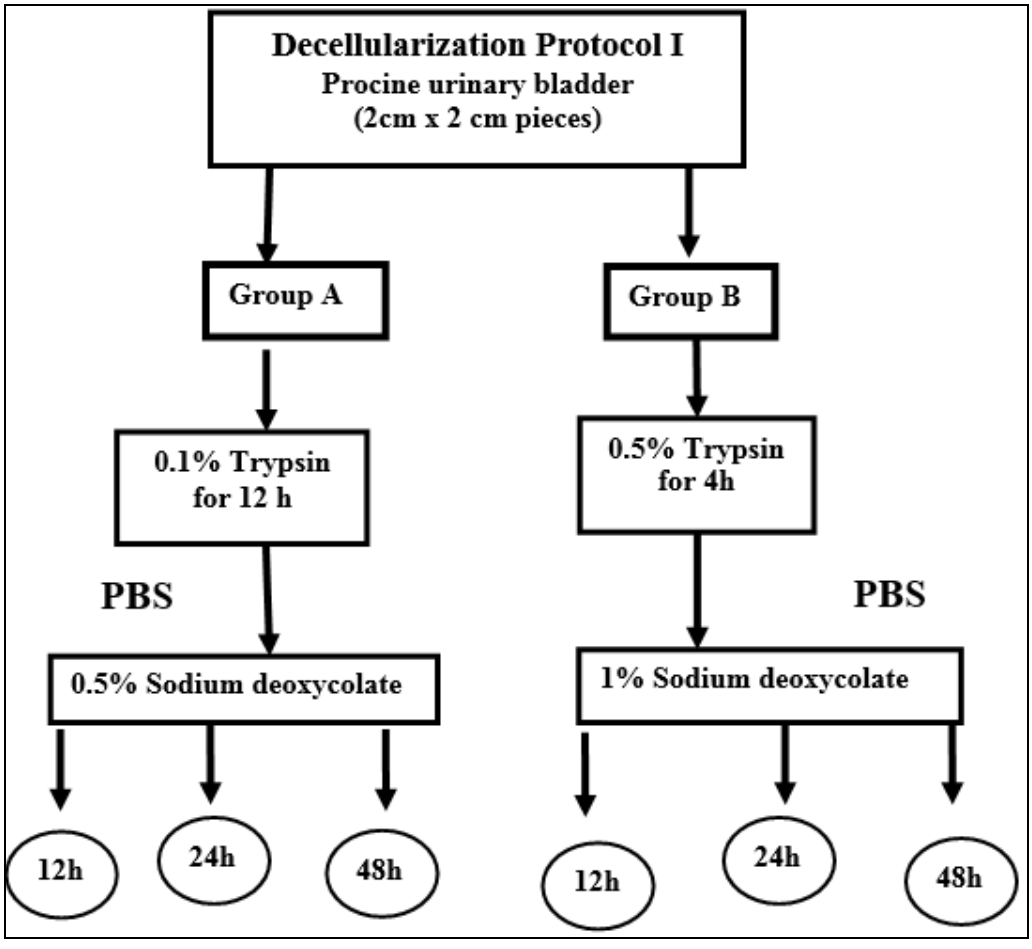
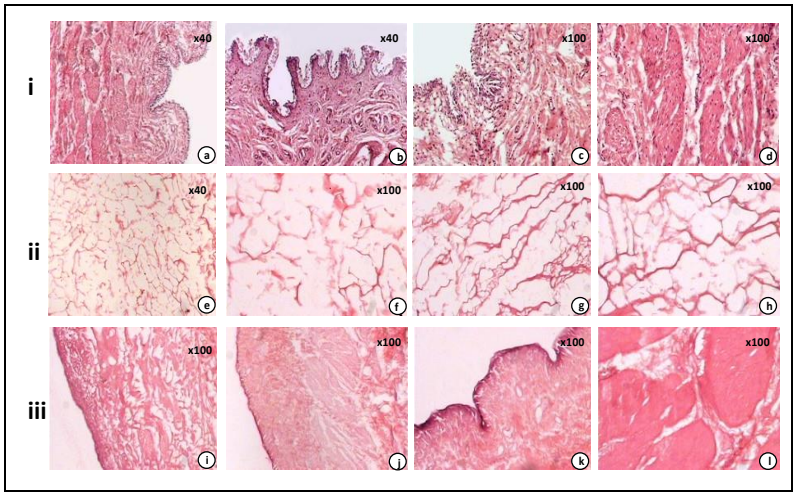


Fig 1: Showing decellularization protocol I in flow chart



1. Normal histoarchitectural detail of porcine bladder (top row of the panel) showing epithelium, lamina propria, submucosa and detrusor muscle layer.
2. Porcine bladder after treatment with trypsin along with different biological detergent (middle row of the panel) showing remnants of the collagen fibres due to excessive dissolution. dodecyl sulfate
3. Porcine bladder after decellularization process with 0.5% Sodium dodecyl sulfate for 24 h (bottom row of the panel) showing intact histoarchitecture of native bladder devoid of cells.

Fig 2: Decellularization of porcine urinary bladder into bladder acellular matrix graft using biological detergents and enzymes

(B) Histopathology of prepared bladder acellular matrix

The prepared bladder acellular matrix graft was preserved for Histopathological examination to judge the efficacy of different protocols used for decellularization of porcine urinary into bladder acellular matrix graft. The prepared graft was fixed in formalin as per standard protocol for Histopathological study. The formalin fixed graft was washed and dehydrated in graded ethanol and embedded in paraffin wax. Fixed graft was sectioned at 5 µm thickness and stained with Haematoxylin and Eosin as per standard method mentioned by Luna [10] for histopathological/microscopic examination**.** The preserved BAM grafts were evaluated on the basis of the histopathological scores recorded in following four parameters.

1. **Basement membrane:** 0=continuous (intact); 1=dis-continuous; 2=present at some places; 4=absent;
2. **Cellularity:** 0=complete acellular; 1=1-30%; 2=30-50%; 3=50-70%;4=>70%;
3. **Collagen fibre arrangement**: 0=compact; 1=mildly loose; 2=moderately loose; 3= heavy loose;
4. **Collagen fibre bundle thickness**: 0= normal; 1=mildly thin; 2=moderately thin; 3=very thin.

Results

The normal histoarchitectural detail of porcine bladder is shown in Figure 2.i (a-d). The decellularization of porcine urinary bladder into bladder acellular matrix graft (BAMG) using different biological detergents and enzymes was done but the results obtained with trypsin and different biological detergents were unsatisfactory. Grossly, bladder acellular matrix graft scaffold prepared by above protocol appeared more spongy and softer than native. The trypsin (0.1%) solution which was used to extract out the cellular contents from urinary bladder wall caused excessive digestion of tissue. The microscopic studies revealed that bladder acellular matrix grafts were almost dissolved and few thread like structures resembling the collagen fibres in structure were seen. With the increase in concentration of trypsin (0.5%) and time interval the graft showed more dissolution and only few remnants of tissue were left as a result these treated tissue samples were not suitable for scaffold (figure 2.ii e-h). Therefore, the protocols were modified to obtain the desirable results. The trypsin treatment which was used before biological detergents was omitted. Therefore, in modified decellularization protocols, tissue samples were directly treated with different biological detergent like Sodium deoxycolate, Sodium Dodecyl Sulfate, TritonX100 and Tween 20 with antibiotic (Cefotaxime) for the preparation of bladder acellular matrix graft.

Modified decellularization protocols

Macroscopic observations

All the bladder tissue samples treated with different concentrations (0.5% and 1%) of Sodium deoxycolate and SDS at 24 h were soft and spongy in consistency and slightly whiter than original tissue. The graft tissue samples treated with similar concentration of Sodium deoxycolate and SDS at 48 h showed similar consistency as that of 24 h treated graft, but with increase in time interval the graft became more slimy, slippery, mucus like and more spongy in consistency. The findings observed in all graft tissue samples treated with different concentrations of TritonX100 and Tween 20 at 48 and 72 h were similar to that of 72 h treated graft with different concentrations of Sodium deoxycolate and SDS. Therefore, macroscopically the graft treated for 48 and 72 h were not suitable to act as scaffold.

Microscopic observations

The histological details of bladder acellular matrix graft prepared after 24 h treatment are presented in Table 1. The porcine bladder on treatment with 0.5% Sodium deoxycolate treated for 24 h showed complete loss of cellularity. The basement membrane over the mildly loose and slightly thin collagen matrix in the sub mucosa was intact. The muscle bundles were also slightly thin in consistency (figure. 3A). The grafts treated with 1% concentration of Sodium deoxycolate showed discontinuous basement membrane. The collagen both in submucosa and between the muscle bundles appeared moderately loose and thin. The graft became completely acellular (figure.3B). In 0.5% SDS treated

bladder, the intact basement membrane was found intact over the loose collagen fibres of the submucosa. The muscles bundles and the collagen connective tissue were slightly loose and thin (figure.3C). The whole graft was acellular. However, grafts treated with 1% concentration of SDS revealed normal basement membrane, collagen fibers in submucosa and muscles. The cellularity was present in muscular layer and serosa (figure.3D). Mildly loosening of collagen fibers was observed. In 0.5% TritronX100, the scaffold showed complete absence of basement membrane. The collagen in submucosa was moderately loose and thin (figure.3E). The muscle fibers, connective tissue between the bundles and the serosa were nucleated (>70%). The scaffolds treated with 1% concentration of TritronX100 revealed the presence of basement membrane at places over the moderately loose and thin connective tissue of the submucosa (figure.3F). However, the muscle bundles, intermicial connective tissue and serosa showed cellularity which was similar to native graft. The grafts treated with 0.5% concentration of Tween 20 showed discontinuous basement membrane over the mildly loose and thin connective tissue without any cellularity. While muscular layer and serosa showed cellularity greater than 70% (figure.3G). However, in 1% concentration of Tween 20 the changes observed in scaffold remained almost same to that recorded in 0.5% concentration of Tween 20 (figure.3H).

The microscopic results of bladder acellular matrix graft prepared after treatment for 48 h are presented in Table 2. Bladder grafts on treatment with 0.5% Sodium deoxycolate revealed complete absence of cellularity. There was moderate thinning and loosening of collagen bundles and muscle fibers (figure.3I). The grafts on treatment with 1% solution of Sodium deoxycolate became acellular. The basement membrane along with sub mucosal connective tissue was absent, while connective tissue between the muscle fascicles were mildly thin and loose (figure.3J). In 0.5% SDS, the change observed in graft was similar to that 24 h treated graft. The grafts were acellular (figure.3K). The grafts on treatment with 1% solution of SDS revealed more than 70% cellularity. The collagen fibres were slightly loose and thin (figure.3L). The scaffold showed similar changes to that 24 h treated graft in 0.5% TritronX100 (figure.3M). On treatment with 1% concentration of TritronX100 the grafts were slightly less cellular compared to 24 h treated grafts (figure.3N). In 0.5% concentration of Tween 20 treated graft, the cellularity was around 30% and there was no basement membrane. Collagen fibers were moderately thin and loose (figure.3O). The grafts on treatment with 1% concentration of Tween 20 revealed discontinuous basement member over the loose collagen. There was complete acellularity within the graft (figure. 3P).

The time interval of biological detergent was increased upto 72 h in modified protocols to obtain the desirable results and microscopic examination of bladder acellular matrix graft prepared by modified protocols are presented in Table 3. In 0.5% and 1% Sodium deoxycolate treated scaffold, the changes remained almost same to that of 48 h treated graft (figure.3Q and 3R). In 0.5% SDS treated graft, smooth muscle fibers had nuclei similar to that in native graft (figure.3S). The grafts on treatment with 1% solution exhibited complete absence of cellularity. The basement membrane was intact over slightly loose and thin connective tissue in submucosa. The muscle fibers and collagen had also reduced in thickness (figure.3T). The changes noticed in 0.5% TritronX100 treated grafts were similar to that in 24 h treated scaffold (figure.3U). The grafts treated with 1% concentration of TritronX100 were slightly less cellular compared to 24 h treated graft (figure.3V). In 0.5% solution of Tween 20 treated grafts revealed no change and therefore resembled the native tissue sample (figure.3W). The grafts on treatment with 1% solution of Tween20 exhibited intact basement membrane over the mildly loose collagen and >70% cellularity within the muscularis layer (figure.3X). In the present study, urinary bladder graft treated with 0.5% SDS for 24 h showed complete acellularity with normal collagen fibres arrangement as shown in Table 1 and Figure 2 iii (i-l). Therefore, in the present study, the modified protocol II using 0.5% SDS for 24 h was found to best for decellularization of porcine urinary bladder into bladder acellular matrix graft.

Table: 1. Modified decellularization protocol at 24h for bladder acellular matrix graft of porcine origin

Detergents Name	**SD**		**SDS**		**TritronX100**		**Tween 20**	
Concentration	0.5%	1%	0.5%	1%	0.5%	1%	0.5%	1%
Basement membrane	0	1	0	0	3	2	1	0
Cellularity	0	0	0	3	4	4	4	4
Collagen fibers arrangements	1	2	1	0	2	2	1	1
Collagen fiber bundle thickness	1	2	0	1	2	2	1	0

Table: 2: Modified decellularization protocol at 48h for bladder acellular matrix graft of porcine origin

Detergents Name	**SD**		**SDS**		**TritronX100**		**Tween 20**	
Concentration	0.5%	1%	0.5%	1%	0.5%	1%	0.5%	1%
Basement membrane	0	3	0	0	3	0	3	1
Cellularity	0	0	0	4	4	3	1	0
Collagen fibers arrangements	2	1	1	1	2	2	2	2
Collagen fiber bundle thickness	2	1	1	1	2	2	1	1

Table: 3 Modified decellularization protocol at 72h for bladder acellular matrix graft of porcine origin

Detergents Name	**SD**		**SDS**		**TritronX100**		**Tween 20**	
Concentration	0.5%	1%	0.5%	1%	0.5%	1%	0.5%	1%
Basement membrane	0	1	0	0	3	0	0	0
Cellularity	0	0	4	0	4	3	4	4
Collagen fibers arrangements	2	1	1	1	2	2	1	1
Collagen fiber bundle thickness	2	1	1	1	2	2	1	1

Histopathological grading: Basement membrane: 0=continuous (intact); 1=discontinuous; 2=present at some places; 4=absent; Cellularity: 0=complete acellular; 1=1-30%; 2=30-50%; 3=50-70%;4=>70%; Collagen fiber arrangement: 0=compact; 1= mildly loose; 2= moderately loose;3= heavy loose; Collagen fiber bundle thickness: 0= normal; 1= mildly thin; 2= moderately thin; 3= very thin.

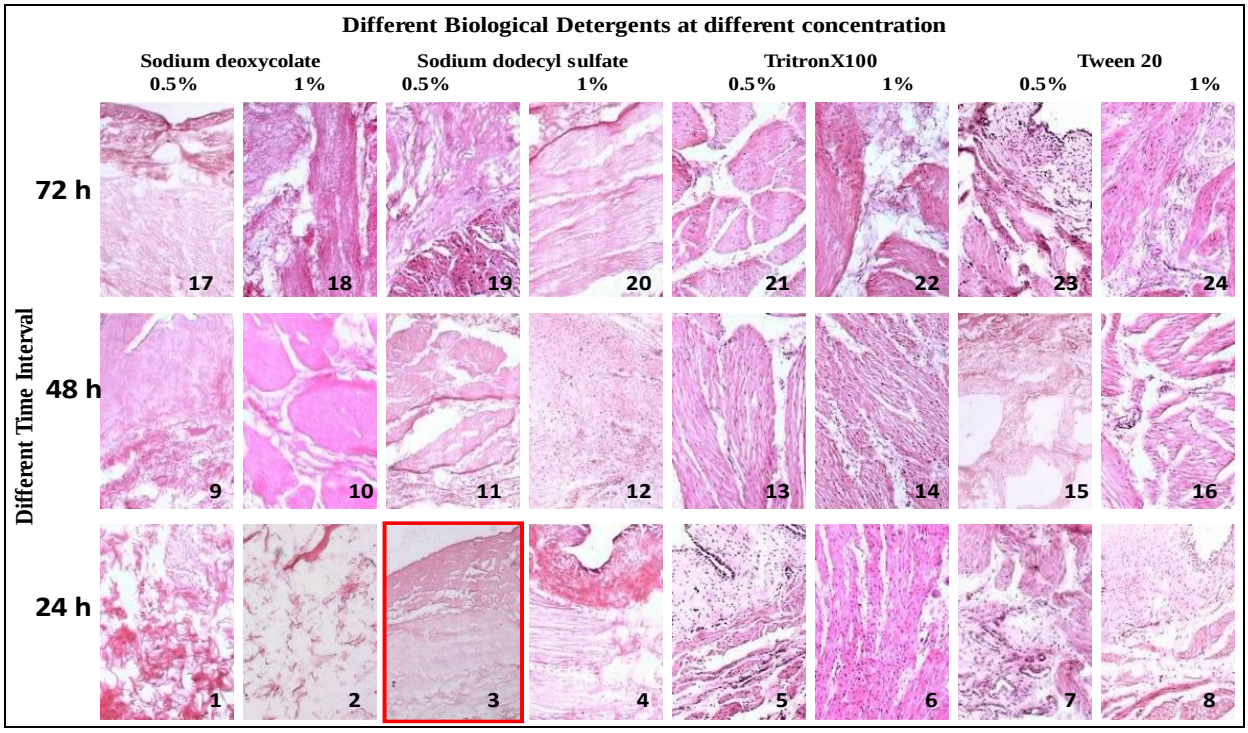


Fig 4: Bladder acellular matrix graft of porcine treated with different concentrations of detergents at different time intervals. The grafts treated for different time intervals were graded on scale 1-30, 30-50, 50-70 and >70-100%. The graft no. 3 showed almost 100% acellularity which was treated with 0.5% sodium dodecyl sulfate for 24 h (HE, X 100)

Discussion

Tissue engineering helps in the development of bladder acellular matrix graft which can be obtained from pig, sheep or other animal for replacement of damaged or diseased bladder tissue. The BAMG can used as scaffold to promote remodeling by creating cell: matrix and even cell: cell interactions. The urinary bladder is a complex organ, whose main functions are storage of urine under low and stable pressure and micturition. There are many clinical conditions, which cause poor bladder compliance and its reduced capacity and require bladder augmentation. Gleeson and Griffith [9] have described the ideal material for bladder should be biocompatible and mechanically reliable, resistant to extraluminal infection but tolerant of intraluminal infection and easy to implant surgically. A common technique in tissue engineering is the use of extracellular matrix (ECM) scaffolds for surgical and wound healing application. ECM scaffolds can promote a constructive remodeling response, including attraction of tissue appropriate cells and formation of healthy tissue. A natural, ECM-based material derived from (xenogenic source) porcine bladder that retains the same physical and structural features as native bladder tissue, but which has had all cellular components capable of producing an immunogenic response removed, should be an ideal candidate for bladder augmentation. While preparing ECM scaffolds, it is important to remove cells and cellular debris which involves a combination of physical methods to delaminate layers of tissue, followed by chemical and enzymatic methods to remove cell bodies from the remaining ECM [8]. If a scaffold has too much cellular material remaining, it can cause problematic immune responses such as chronic inflammation. Conversely, if the scaffold is depleted of growth factors or if mechanical properties of the scaffold are too altered, constructive tissue remodeling will not occur. Establishing a criteria for decellularization process is critical while preparing ECM scaffolds. Such decellularization strategies, designed to limit the immunogenicity of the matrix whereas, remnants of cell components in xenografts may contribute to calcification and/or immunogenic reaction [5]. In the present investigation, decellularization process removes the nucleus and cytoplasmic cellular components successfully, resulting into full-thickness bladder acellular matrix graft (BAMG) of porcine origin consisting of primarily collagen and elastin which have good tensile strength. Similarly, Courtman *et al*. [5] also mentioned that decellularization process effectively remove nucleus and cytoplasmic cellular components, lipid membranes and membrane-associated antigens as well as soluble proteins, while preserving the original structural arrangement of extracellular matrix components which consist of primarily of insoluble collagen and elastin fibres which are embedded in a ground substance of glycosaminoglycan's. The acellular tissue matrices must be biocompatible, slowly degraded upon implantation and replaced and remodeled by the extracellular matrix proteins synthesized and secreted by ingrowing host cells, which reduced the inflammatory response [12]. Yoo *et al.* [20] also reported that the acellular matrices supported the regeneration of tissues with no evidence of immunogenic reaction. However, it was important to keep in mind that even after the removal of cells and cell debris, the intact extracellular matrix of the acellular tissue itself might have elicited an immune response as mentioned by Coito and Kupiec-Weglinsky [4].

In the present study, first decellularization of porcine urinary bladder into acellular matrix graft was done by using the combination of trypsin with different biological detergents in different concentrations. But the result with this protocol was unsatisfactory. The microscopic examination of acellular matrix grafts obtained after treatment with 0.1% trypsin showed few thread like structures resembling the collagen. With the increase in concentration of trypsin (0.5%) and time interval the graft showed more dissolution and only few remnants of tissue were seen, as a result these treated tissue

samples were unfit to act as a good scaffold. This might be due to excessive digestion of the bladder tissue by trypsin. However, Yang *et al*. [19] prepared rabbit bladder extracellular matrix (BECM) by keeping bladder in 0.4% trypsin for 5-6 h at 37 ^{0}C which was followed by the treatment with DNase for 6-8 h at 37 ^{0}C along with 4% sodium desoxycholate for 5-6 h. But in our study, prepared graft by using trypsin was unsatisfactory as per histopathological scores. Therefore, decellularization protocols were modified and only biological detergents in different concentrations viz., Sodium deoxycolate, Sodium Dodecyl Sulfate, TritonX100 and Tween 20 were used to treat bladder tissue for different time intervals. After each treatment bladder acellular matrices were washed with PBS to remove any residual chemical and biological agents. Histological sections were examined by light microscope to confirm the acellularity of the BAMG with normal collagen fibres arrangement. Histopathologically, bladder graft treated with 0.5% and 1% Sodium deoxycolate and Sodium Dodecyl Sulfate (SDS) for different time intervals showed complete acellularity whereas tissue treated with 0.5% and 1% TritonX100 and Tween 20 did not reveal complete acellularity and >70% cellularity were seen. The muscle fibres, connective tissue between the bundles and the serosa were nucleated (50->70%). On the basis of the histological observations, Sodium deoxycolate and Sodium Dodecyl Sulfate (SDS) were found to be best for decellularization of porcine urinary bladder into bladder acellular matrix graft and the graft showed an intact mesh with no evidence of cells, nuclei or other cell fragments. The prepared BAM graft had shown that underlying bladder histoarchitecture was also retained. Similar decellularization process also used by Geng *et al*. [7] for preparation of xenogenic bladder submucosa acellular matrix (BSAM) by using only biological detergent 0.5% SDS and dH_2O. Rosario *et al*. [17] also proved that Sodium dodecyl sulfate (SDS) is highly effective decellularizing agent for porcine urinary bladder. In the present study, the grafts treated with 0.5% and 1% Sodium deoxycolate became completely acellular with mildly loose and slightly thin collagen fibres arrangement. The prepared bladder acellular matrix graft treated with 0.5% Sodium Dodecyl Sulfate (SDS) for 24 h provided better acellularity with normal collagen fibres morphology of porcine bladder. The BAM graft retained the physical and structural features of the native bladder tissue and contained no residual cells or nuclear bodies. Several workers had prepared bladder acellular matrix graft with 4% sodium deoxycholate and DNase combination [18, 15, 14]. Merguerian *et al*. [11] and Reddy *et al*. [16] also optimized the protocol for preparation of bladder acellular matrix graft by treating porcine bladder using sodium dodecyl sulfate (SDS) extraction method. The whole bladder was placed in a Trizma/EDTA solution for 48 h and followed by overnight incubation with DNase and RNase solutions, and suspended in a Tris/SDS solution for 48 h. Brown *et al*. [2] obtained bladder acellular matrix by using sodium dodecyl sulphate residual surfactant for 24 h with a pH 9 tris solution. The acellularity was evaluated using histology and IHC to confirm the effectiveness of the extraction process. Whereas, Bolland *et al*. [1] developed full-thickness porcine bladder acellular matrix by using hypertonic buffer containing 0.1% (w/v) SDS and nuclease enzymes.

Conclusion

From the present study, it could be concluded that the decellularization of porcine urinary bladder into bladder acellular matrix graft was successful optimized and treatment with 0.5% SDS concentration for 24 h resulted in complete acellularity with retention of normal bladder histoarchitecture which revealed that there was an intact structure of collagen and elastin matrix with no evidence of nuclei which could be used as scaffold for bladder tissue engineering.

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