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Preparation of acellular diaphragmatic scaffold of bubaline origin

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Abstract
A modified method for decellularization of buffalo diaphragm was developed to prepare an acellular biological scaffold which can be utilized for repair of varied muscular defect in both large and small animals of different species. The method allowed an efficacy of 94.61 % for decellularization when 1% SDS was used as detergent. Histology, nuclear staining and gel electrophoresis confirmed the acellular nature of scaffold. Keeping quality of scaffold was excellent even after storage of 12 months before clinical use. Such material will reduce the dependency over tissue donor and percentage of graft rejection.

Keywords: Buffalo, decellularization, DNA quantification, Scaffold, SDS

1. Introduction
The clinical need for strong and biocompatible materials that persuade amalgamation while minimizing adverse reaction, such as tissue reaction and adhesion formation, is apparent. The design of most advantageous surgical repair materials to strengthen or reinstate soft tissue remains awkward. When normal tissue fails, surgeons are confronted with the challenge of manipulating available healthy anatomy in such a manner as to produce normal form and function in the affected region with minimum tissue loss [3].
Synthetic and biological materials are used as prosthesis for these replacements particularly for the repair of large abdominal wall defects in animals where a substantial loss of thick muscular compartment had reduced the strength and made spontaneous healing a time consuming and uneconomic process. The diverse synthetic materials used are polyester fabric, nylon, dacron, stainless steel, cotton mesh, mosquito net mesh, vicryl mesh, polypropylene mesh [8], expanded poly tetrafluoro ethylene (PTFE), marcelene mesh, carbon fibers, carbon sheet, oxidized generated cellulose and polyethylene glycol [1]. A synthetic non–absorbable material polypropylene mesh is the most extensively used material for abdominal wall substitution and strengthening during hernia repair [4].
In the surgical repair of congenital abdominal wall defects, the easy availability of a nonimmunogenic and non-prosthetic biomaterial that could guide the regeneration of normal tissue is a fascinating possibility. Biomaterials are already in use, but an acellular matrix (ACM) can arouse exact regeneration of the mislaid tissue. Decellularized scaffolds can be prepared from animal tissues and represent a promising biomaterial for exploration in tissue regeneration studies [13]. The natural bio-scaffold have advantages over synthetic materials that they impersonate natural extra cellular matrix (ECM) formation and composition, imitate natural stimulatory effects of ECM on cells and permits the merger of growth factors and other matrix proteins to further enhance cell functions. The ultimate goal of any decellularization protocols is to remove all cellular material without adversely affecting the composition, mechanical integrity and eventual biological activity of the remaining ECM.
Present investigation was therefore undertaken to investigate the efficacy of decellularization protocol for buffalo diaphragm.

2. Materials and Methods
2.1 Preparation of decellularized bubaline diaphragmatic scaffold
Ten pieces of fresh buffalo diaphragm were collected in phosphate buffer saline (PBS) supplemented with antibiotics i.e. gentamicin @ 80µL/ml, from the local slaughter house. All the blood clots and visible contaminants were removed by thorough cleaning in sterile PBS. The diaphragmatic tissues were cut to the size of 2 X 2 cm.

These were decellularized by using method of Kumar *et al.* [9] with slight modifications. After cutting these were placed in sterile PBS approximately for 2 hours at room temperature. The diaphragmatic tissues were placed in 1% sodium dodecyl sulphate (SDS) solution and kept in an orbital shaker at a rotation speed of 50 rotations/min at 21 ˚C for 12 hours. Later on, tissues were washed three times in sterile PBS and again placed in freshly prepared 1 % SDS solution. Tissues were placed in orbital shaker for next 12 hours at 50 rpm and 21 ˚C for next 12 hours. This procedure was repeated for next 48 hours (total three days). Thereafter, tissues were washed four times in sterile PBS and placed in high grade 70% ethanol in orbital shaker for three hours at 21 ˚C, 20 rpm. Tissues were taken out and washed in sterile PBS for three times and again placed in orbital shaker at 21 ˚C, 20 rpm, for three hours to remove residual ethanol. Tissues were finally rinsed in fresh PBS and stored in their flat position with minimum quantity of sterile PBS at -20 ˚C till use. In this protocol we utilized 1% SDS as a decellularization agent instead of using different concentrations of sodium deoxycholate utilized by Kumar *et al.* [9]. Besides this, minor modifications in terms of processing of samples were also made.

2.2 Evaluation of prepared decellularized scaffold before application

2.2.1 Histology

Diaphragmatic tissue was embedded in paraffin wax after being fixed in 10% buffered neutral formalin. Embedded tissues were sectioned at a thickness of 5 µm along the perpendicular direction of the fibers. H & E staining along with Masson's Trichome staining (for collagen fibers) were done to visualize the cellularity and orientation of collagen fibers.

2.2.2 4,6-Diamino-2-phenylindole dihydrochloride (DAPI) staining

For staining of nucleus DAPI staining was done as per standard protocol in diaphragmatic tissues before and after decellularization. Working DAPI stain (1µg/ml) was prepared by adding double sterile PBS to stock solution (1mg/ml). The slides having thin deparaffinized samples were smeared with working DAPI solution and incubated for 15 minutes in a light proof room. Excess solution was drained and slides were washed many times with sterile PBS. The stained slides were viewed using a fluorescent microscope with appropriate filters.

2.2.3 DNA isolation and quantification

DNA quantification was done as per method described by John *et al.* [7] with some modifications. For this, exactly one gram of diaphragmatic tissue (before and after decellularization) from each diaphragm was weighed and triturated using pestle and mortar in liquid nitrogen till it became fine powder. Then this powdered tissue was transferred in a 15 ml sterile tube and mixed in 2 ml of solution-1 by continuous shaking for 10 minutes. Then it was centrifuged at 5000 rpm for 10 minutes and supernatant was discarded. The sediment was mixed in 400 µl of solution-2 and 2 µl of proteinase K in a 2 ml sterile tube and maintained in a warm water bath at 56 ˚C for 12 hours. Then 1 ml of mixture of phenol, chloroform and isoamyl alcohol at a ratio of 25:24:1 was mixed in this and again centrifuged at 8,000 rpm for 15 minutes. By using supernatant, it was mixed in 400 µl of solution containing chloroform and isoamyl alcohol at a ratio of 24:1 and centrifuged at 11,000 rpm for 2 minutes. Supernatant was separated and equal volume of chilled isoamyl alcohol was added in it and it was kept at -20 ˚C for and 10 minutes.

Then it was centrifuged at 10,000 rpm for 15 minutes and supernatant was discarded. The remaining pellet at the bottom of the tube was mixed 1 ml of high grade 70% ethanol and centrifuged at 10,000 rpm. This procedure was repeated for two more times. The resulting pellet was dried by keeping the tube open in the environment till all the alcohol was evaporated. Then this pellet was dissolved in 200 µl of 0.3 X TE buffer by mixing it properly. Then the optical density was measured against the blank (TE buffer) at a wavelength of 260 nm and DNA was quantified using Nanodrop ND-1000 spectrophotometer V3.5 and expressed in terms of ng/µl. Average of DNA content before and after decellularization was calculated.

2.2.4 Agarose Gel Electrophoresis

Exactly weighted 0.5 g agarose powder was mixed in 70 ml of TAE buffer and melted. Melted agarose was poured into sealed gel casting tray with the comb positioned appropriately. Once the gel got solidified, it was transferred to electrophoresis tank filled with 1X TAE buffer. The level of buffer was kept at least I cm above the gel. The wells were carefully charged with combination of 2 µl of of DNA loading dye (Ethidium bromide) and 8 µl of DNA sample in each well. Electrophoresis was carried out first at 100 V for 5 minutes followed by 70 V for next one hour. On completion of process, the gel was visualized under UV light and documented by Geldoc® gel documentation system and presence or absence of DNA in samples after and before decellularization was judged.

3. Results and Discussion

Recipients of an allogenic and more specifically xenogenic scaffolds based on extracellular matrix may encounter adverse immunogenic response due to foreign antigenic epitopes related to intracellular organelles and cell membranes. This has to be eliminated for their acceptance in the biological systems [12]. This antigenicity of xenogenic biomaterials can be reduced by a variety of chemical and physical methods. Chemicals, such as acid solutions, hypotonic/hypertonic solutions, detergents, organic solvents, and enzyme solutions tend to disrupt the cellular and extracellular components and their antigenic epitopes. Gamma radiations, sonication and freezing thawing are commonly used physical modalities to decellularize the biological tissues and these are most of the times used in conjunction with chemical treatment [5].

The tendinous portion of an adult buffalo diaphragm is a thick piece of collagen fibers oriented in multiple direction covered with peritoneum from abdominal side and with pleura from thoracic side. This provides a rigid configuration and greater weight bearing ability. It is also elastic in nature and can be stretched up a certain extent due to elastin and reticulin content. These advantages of buffalo diaphragm were utilized for preparation of a decellularized scaffold which on application can with hold its mechanical properties and is resistant to tearing due to over weight of large animals where conventional meshes (nylon, polypropylene, pogalactin, proline etc) can't withstand.

Detergents are molecules with exclusive properties of alteration, in form of either disintegration or synthesis, of hydrophobic – hydrophilic interactions among molecules in

biological tissues. Here we utilized cell lysing and protein solubilization property of SDS. SDS is an anionic detergent which acts on membrane proteins (hydrophobic) as well as nonmembrane (hydrophilic) proteins. At its higher concentration (more than 2%) increased pH (alkaline) and temperature (above 50 ˚C) of solution, it tends to denature the conformation of collagen. Here we kept the pH of the solution towards neutral and maintained 21 ˚C temperature in orbital shaker to avoid undue damage to ultrastructure of collagen. Also the concentration of solution was maintained at 1%, for this solution was replaced with freshly prepared solution of 1% SDS after each 12 hours up to three days. Zhou *et al.* [15] also used 1% SDS for decellularization of bovine pericardium and reported that it preserved the ECM components and architecture. Youngstorm *et al.* [14] reported no alteration in ultimate tensile stress and maximum stress in decellularized equine superficial digital flexor tendon using 2% SDS. However, glycosaminoglycan (GAG) content was reduced. The colour of diaphragmatic tissue changed from pale yellow (before treatment) to milky white after treatment (Fig. 1).

Modifications in the original protocol given by Johns *et al.* [7] for DNA isolation from blood were found appropriate for isolation of DNA from diaphragmatic tissue. The tissue was triturated in liquid nitrogen which provided a homogenous mixture of tissue and dissolving solution without any solid tissue remaining at the bottom of the tube. DNA isolation from this method resulted in intact DNA without RNA contamination and final DNA preparation was translucent to slightly whitish in colour. The isolated DNA was easily dissolved in standard TE buffer. The quantity of DNA, obtained from untreated and decellularized diaphragmatic tissue was easily comparable. The mean quantity of DNA in untreated samples (n=12) were 952.20±25.43 ng/µl which significantly (p<0.01) reduced to 51.31±4.24 ng/µl in decellularized diaphragmatic tissue samples, indicating 94.61% efficacy of decellularization protocol (Table 1). Lin *et al.* [10] obtained 86.4% DNA clearance by using SDS along with trypsin and DNAase. As it is not possible to remove all immunogenic materials (here, DNA) but, the residual cellular antigens may be insufficient to elicit the type of proinflammatory or immune response that could adversely affect biologic scaffold remodeling [11]. It is also possible that the chemical decellularization has altered the protein of reactive antigens in such a manner that it can no longer stimulate the adverse reaction.

Table 1: Mean values of DNA concentration (ng/µg) in diaphragmatic tissue before and after decellularization

Sample No.	Before decellularization	After decellularization
1	1076.3	59.7
2	1030.4	60.4
3	1058.0	51.3
4	956.3	52.3
5	843.2	45.3
6	963.8	48.3
7	1003.5	58.1
8	974.2	49.2
9	890.1	48.2
10	780.0	58.4
11	890.4	42.2
12	960.3	49.2
Mean± SE	952.2±25.41	51.11±1.72
t –value	36.29**	

** Significant (p<0.01)

Agarose gel electrophoresis revealed a thick white band over gel indicating presence of nuclear content in untreated tissue samples. However, gel images of DNA samples from decellularized diaphragmatic tissues revealed only a smear without any detectable bands indicating complete degradation of DNA in the tissue (Fig. 2). Similar findings are also reported by Andrea *et al.* [2] during decellularization of skeletal muscles of rat, rabbit and human.

Histological evaluation of tendinous portion of buffalo diaphragm revealed that cellularity was intact in diaphragm before treatment as suggested by H&E, Masson's Trichome and DAPI stained sections but treatment with 1% SDS for 72 hours resulted in loss of cellularity without affecting the three dimensional structure of collagen fibers (Fig. 3,4 and 5). The chemical process of decellularization resulted in a sheet of homogenous extracellular matrix consisting mainly of collagen and also elastin and reticulin, and removed all soluble proteins in the matrix. These findings are in consonance with the study of Lin *et al.* [10] and Kumar *et al.* [9]. The clearance of nuclear content was further evident in DAPI staining. These findings are in accordance with the study of Gilbert *et al* [6] who find SDS as an effective agent for decellularization of biological tissue. However, Kumar *et al.* [9] observed that 2% sodium deoxycholate was effective for decellularization of bubaline diaphragm.

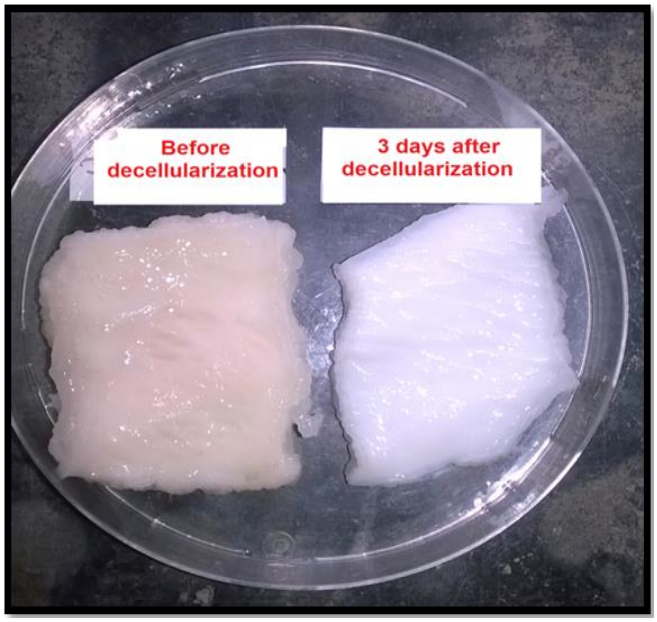
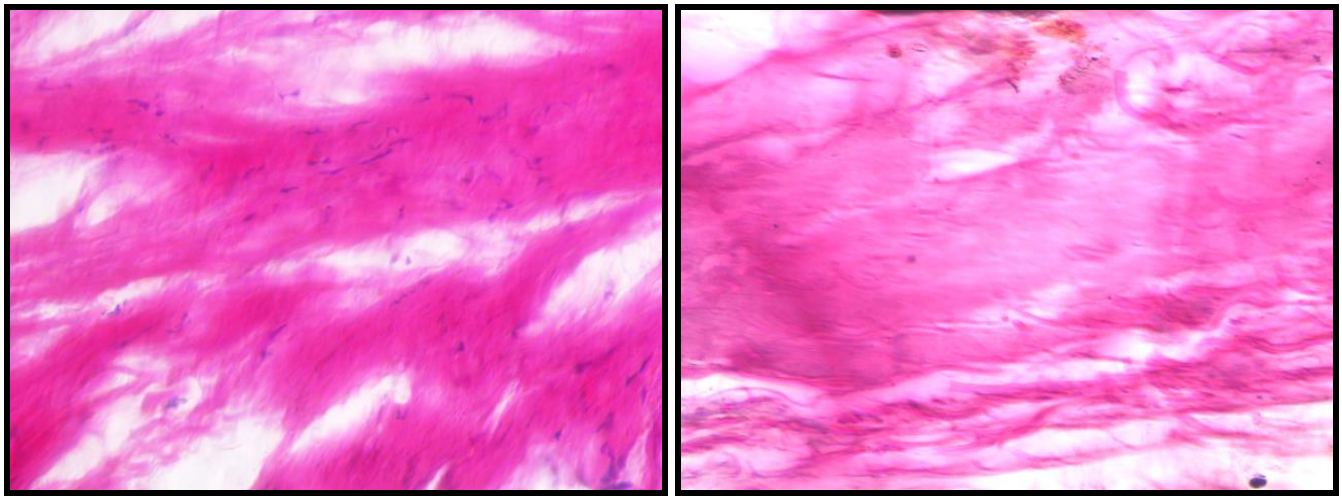


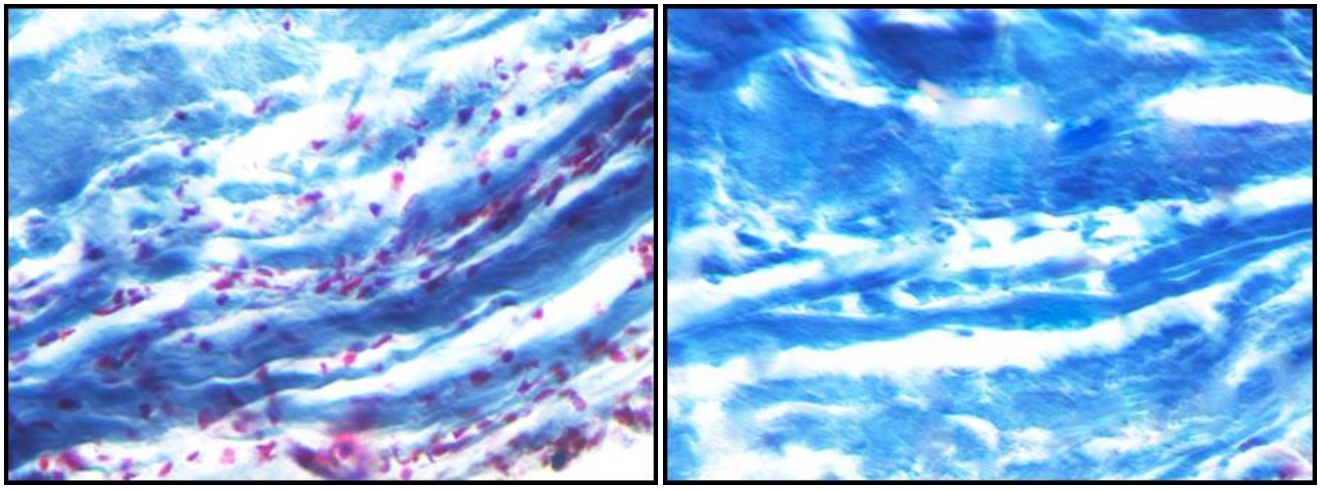
Fig 1: Diaphragmatic tissue before and after decellularization

Fig 2: Imaging of DNA samples by gel documentation systems after agarose gel electrophoresis of untreated diaphragmatic tissue (1), decellularized diaphragmatic scaffolds (2-8)



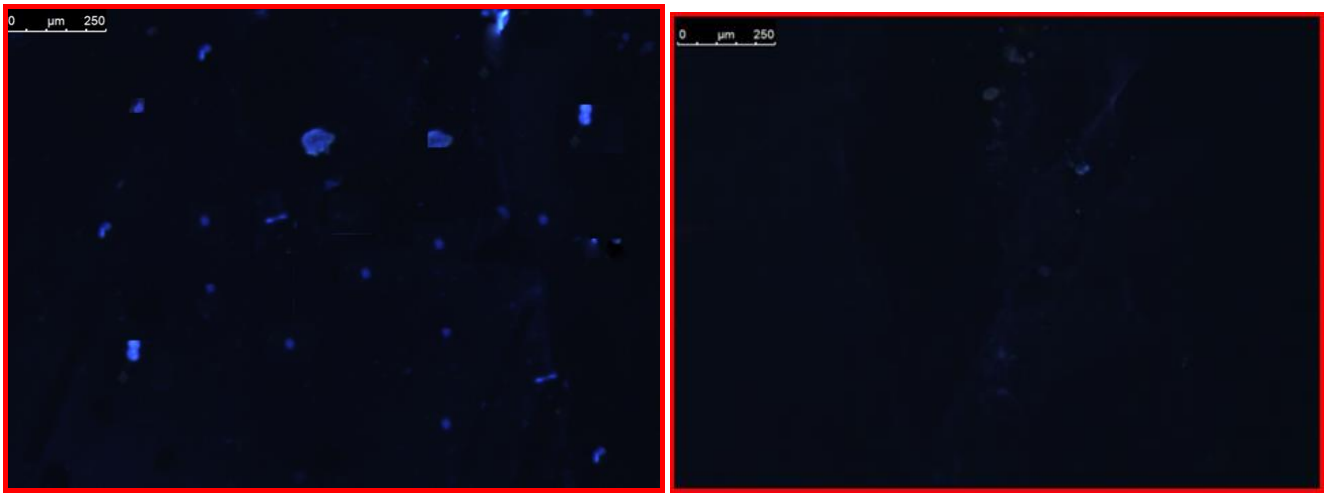
(a) (b)

Fig 3: Section of diaphragmatic tissue before decellularization. (a) Nuclei visible, (b) Nuclei absent. (H & E, 40X)



(a) (b)

Fig 4: Section of decellularized diaphragmatic tissue showing collagen fibers. (a) Nuclei are visible, (b) Nuclei absent. (Masson Trichome, 40X)



(a) (b)

Fig 5: (a) Section of decellularized diaphragmatic scaffold giving fluorescence indicating presence of nuclei, (b) No fluorescence indicating absence of nuclei (DAPI, 40X)

4. Conclusions

The findings of present study suggest that treatment with 1% sodium SDS for 72 hours results in complete decellularization of bubaline diaphragm and matrix which is produced can be used for repair of various soft tissue defects in small and large animals without any fear of life threatening immunological reaction and graft rejection. Xenogenic nature of scaffold also reduces enslavement on availability of autologous and allogenic tissue materials. However, further research in defensible to authenticate the immunological properties of

scaffold. Also, use of stem cells seeded scaffold might be encouraged to accelerate the healing process.

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