Review | Received 26 June 2023; Accepted 20 October 2023; Published 26 October 2023
https://doi.org/10.55092/bm20230008

Bubaline tissues-based extracellular matrix scaffolds for tissue regeneration: current use and future prospectives

Naveen Kumar[1,*], Vineet Kumar[2], Anil Kumar Gangwar[3], Sonal Saxena[4], Pawan Diwan Singh Raghuvansi[5], Dayamon D. Mathew[6], Sameer Shrivastava[7], Aswathy Gopinathan[8]

[1] Veterinary Clinical Complex, Apollo College of Veterinary Medicine, Agra Road, Jaipur-302031, Rajasthan, India
[2] Department of Veterinary Surgery and Radiology, College of Veterinary & Animal Sciences, Kishanganj-855107, Bihar Animal Science University, Bihar, India
[3] Department of Veterinary Surgery and Radiology, Former Registrar and Deputy Director Research, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya-224 229, Uttar Pradesh, India
[4] Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute [Deemed University], Izatnagar-243122, Uttar Pradesh, India
[5] Department of Veterinary Surgery and Radiology, College of Veterinary Science and Animal Husbandry, NDVSU, Mhow-453446, Indore, Madya Pradesh, India
[6] Department of Veterinary Surgery and Radiology, Faculty of Veterinary and Animal Sciences Banaras Hindu University, Rajiv Gandhi South Campus, Barkachha, Mirzapur-321001, Uttar Pradesh, India
[7] Bioassays & Biosensor Laboratory, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute [Deemed University], Izatnagar-243122, Uttar Pradesh, India
[8] Division of Surgery, ICAR-Indian Veterinary Research Institute [Deemed University], Izatnagar-243122, Uttar Pradesh, India

* Correspondence author; E-mail: naveen.ivri1961@gmail.com.

Abstract: Most of the collagen scaffolds used in tissue engineering belongs to porcine or bovine origin. Bovines are prone to different zoonotic diseases like bovine spongiform encephalopathy or mad cow disease, transmissible spongiform encephalopathy and foot and mouth disease. The collagen scaffolds developed from porcine origin has little clinical acceptance in our country on cultural and religious grounds. More over the slaughtering of cattle is illegal/ban in most of the part of India. So, we must look the other sources of the collagen. The collagen scaffolds developed from bubaline (buffalo origin) allow greater clinical acceptance on cultural and religious grounds in addition to presenting a low risk with respect to viral pathogens and prions. Therefore, in the present study we developed collagen scaffolds from bubaline (buffalo origin) species. The protocols for decellularization of

collagen scaffolds from buffalo aorta, diaphragm, rumen, reticulum, omasum, skin, gall bladder and pericardium have been optimized. The developed acellular collagen matrices were preclinically tested in laboratory animals for their biocompatibility. Suitably found matrices were treated clinically in different species of animals for reconstructive surgery. We succeeded in preservation of the complex composition and three-dimensional ultrastructure of the ECM. No methods of decellularization resulted in disruption of the architecture and loss of surface structure and composition. The acellular ECM scaffolds can create a favorable regenerative microenvironment, promote tissue-specific remodeling, and act as an inductive template for the repair and functional reconstruction. In the current review, we will provide a critical overview of the structure and function of various types of extracellular matrix derived from bubaline (buffalo origin), the construction of three-dimensional extracellular matrix scaffolds, and their tissue engineering applications, with a focus on translation of these novel tissue engineered products to the human applications. We will also present an outlook on future perspectives of the extracellular matrix derived from bubaline (buffalo origin), in tissue engineering and regenerative medicine.

Keywords: Bubaline tissues; extracellular matrix; scaffolds; tissue regeneration; preclinical evaluation; clinical evaluation

1. Introduction

Extracellular matrix (ECM) prepared after removing cellular components from native tissues are currently being used to facilitate wound healing and tissue regeneration. These matrices provide a native framework for cell adhesion at the site of a tissue defect and allow local cells to migrate into the matrix, proliferation and adhere before undergoing differentiation. In general, ECM-based biomaterials represent lipid-free, decellularized protein-based derivatives and purified protein extracts of previously living tissues or organs. This final form differentiates the extracted ECM as a biomaterial compared to its living tissue precursor representing an organ or tissue graft for transplant [1–5]. As a completely natural material, the ECM has been proposed to be immune-privileged in the sense that, as a natural material, ECM may not succumb to the typical implant fate and series of host reactions to foreign bodies [6–8]. The logic driving this idea is that naturally derived matrix materials represented by the ECM present naturally derived biomolecular designs and architecture and biological compositions to interrogating host cells after implantation that attenuate the foreign body reaction [9].

Extracellular matrices are slowly degraded by cellular proteases at the implantation site and are replaced by new endogenous extracellular matrix proteins secreted by ingrowing fibroblasts. Further, the matrices stimulate rapid neovascularization during tissue regeneration [10], and are relatively immunologically inert [7]. They are resistant to adhesions and encapsulation [11]. Infection rates of extra cellular matrices scaffolds are relatively low [12], a feature that may be attributable to their inherent antimicrobial activity [13], and/or their ability to rapidly vascularize and therefore clear bacteria. Preservation of the native ultrastructure and composition of ECM during the process of tissue decellularization is highly desirable [14–16]. A biomaterial-based scaffold plays a fundamental role in tissue

engineering because it provides mechanical property and structural support for cell attachment and tissue development, creates a permissive environment for cell survival, proliferation, and differentiation, and eventually promotes tissue regeneration [17,18].

Among various materials used in tissue reconstruction, acellular matrices have been recently utilized [19]. Acellular matrices are the noncellular part of a tissue and consist of proteins such as collagen and carbohydrate structures secreted by the resident cells. They can be transplanted without rejection and provide a conductive environment for normal cellular growth, differentiation, and angiogenesis and a framework for tissue regeneration because they are completely replaced by the host tissue [19]. An ideal scaffold needs to have many specific architectural, mechanical, physicochemical, and biological properties. Firstly, the scaffold should have a porous spongy structure to facilitate cell adhesion and migration and to stimulate angiogenesis and metabolic exchange. Secondly, the scaffold should have a certain shape stability and intrinsic mechanical property that are like that of defect tissues. Thirdly, the scaffold needs to be biocompatible to the body and biodegradable with a controllable rate in the body. Fourthly, the scaffold needs to show no or low immunogenicity. Finally, the scaffold needs to be able to include biological and/or physical cues that affect cell phenotype and promote directed cellular regrowth [20,21]. More importantly, emerging studies suggest that the extracellular matrix can itself function as an inductive scaffold or modify a biomaterial-based scaffold for tissue and/or organ regeneration. Therefore, the extracellular matrix is a critical element in the field of tissue engineering and regenerative medicine [22].

By presenting physically and biochemically “native” matrices to implants sites, ECM biomaterials are proposed to rapidly re-establish healing cues and limit foreign body reactions. Nonetheless, despite a significant history of ECM development, analysis and implant use for decades, currently little consensus exists regarding its ultimate capabilities in modulating host reactions. Certainly, host privilege with regards to minimizing the foreign body reactions and improving implanted materials performance has not been unequivocally demonstrated. The ECM biomaterial utility in implanted forms and its eventual progress in biomedical applications will rely on improved knowledge of compositional identity of decellularized natural materials and how these factors influence host recognition and ultimate implant integration, regeneration and healing. The ECM materials have been traditionally prepared from native tissues of porcine species. However, its biomedical uses are associated with an ethno-cultural stigma [23]. Native tissues of bubaline origin such as aorta, diaphragm, rumen, skin, reticulum, omasum, gall bladder and pericardium may serve as economical, viable, and safer alternative for collagen-rich matrices to mammalian sources. Moreover, the environmental issue related to pollution from these wastes can also be addressed [24–31].

This review examines methods to process tissue explants to yield various ECM materials of bubaline origin, assess their composition and then validate their use as biomaterials in preclinical implant models and finally clinical applications. Understanding the critical performance issues has direct implications on efforts regarding translation of these materials into commercial medical products.

2. Preparation of acellular aortic matrix scaffold

Fresh aorta from deceased donor buffalo was decellularized by treatment with 1% sodium dodecyl sulfate (SDS) detergent for 24 h followed by treatment with 0.25% trypsin enzyme solution for 2 h and then again with same 1% SDS for again next 24 h showed complete acellularity with normal thickness and arrangement of collagen fibers [32,33]. Native and decellularized aortae were examined for residual cells, and collagen structure and integrity by histological examination and scanning electron microscopy (SEM) [34]. Masson's trichrome and Weigert's staining, DNA quantification, and Fourier transform infrared (FTIR) spectroscopy were used for further characterization of the decellularized aorta [35]. Histologically, absence of cells and orderly arranged collagen fibers were observed in the decellularized aorta (Figure 1a,b). The SEM observations confirmed preservation of collagen structure and integrity in the decellularized aorta (Figure 2a–c). Residual DNA content was significantly lower in the decellularized aorta as compared to the native aorta (Figure 3). The DNA extracts from decellularized aorta showed marked removal of DNA material, with absence of DNA band in agarose gel. The FTIR spectroscopy confirmed the presence of organic collagen amide A, amide B, amide I, amide II and amide III functional groups within decellularized aorta [35].

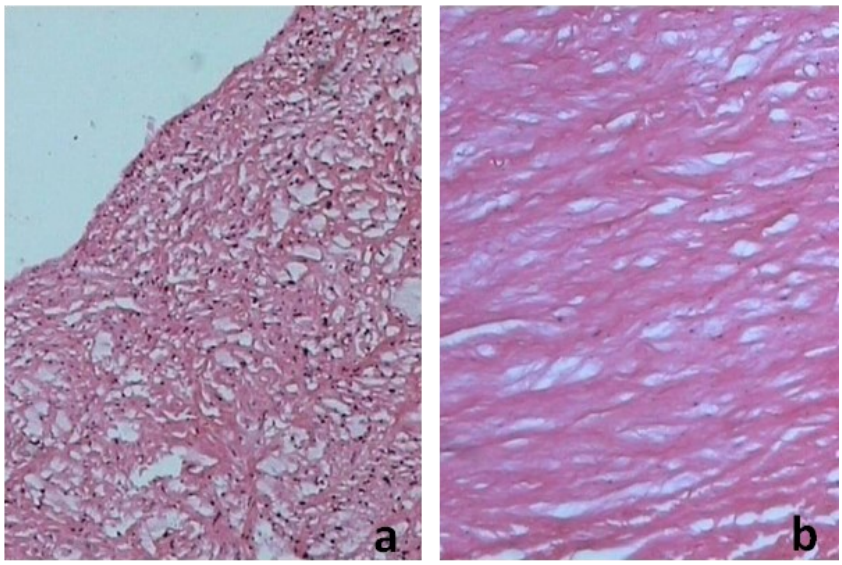

Figure 1. (a)–(b) A histological image of native aorta, b-acellular buffalo aortic matrix [33].

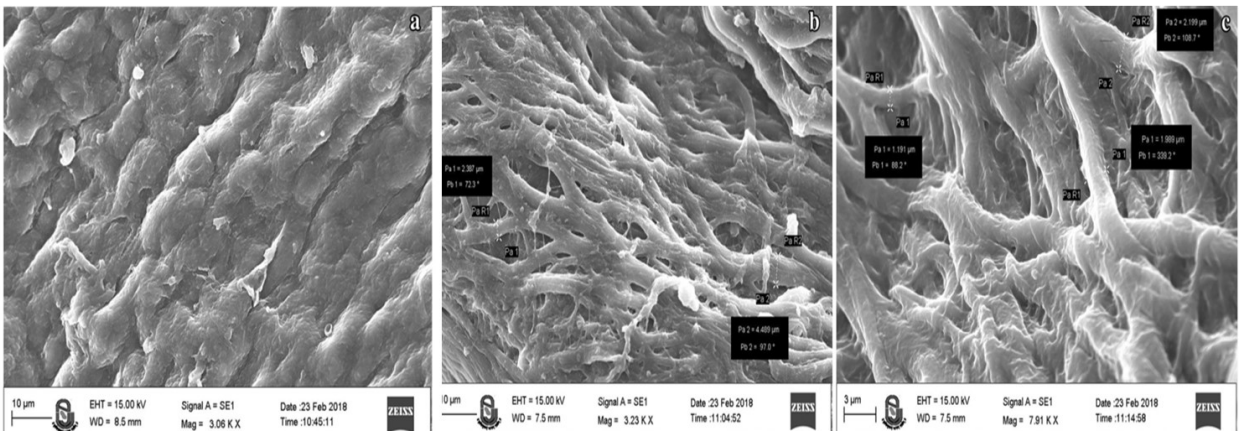

Figure 2. (a) SEM images of native aorta (NA); **(b)** and **(c)** acellular buffalo aortic matrix (BAM) [35].

Each layer of aorta contributes uniquely to the overall mechanical properties. Collagens types I and III are major collagens in blood vessels. The great strength of collagen fibers, however originates from the stable intermolecular covalent bonds between adjacent tropocollagen molecules [36]. The goal of any decellularization protocol is to effectively remove immunogenic cellular material while maintaining the biological activity and mechanical integrity of the ECM. Through decellularization procedures vascular tissue can be reduced to a sterilized scaffold and implanted with a low risk of rejection. The treatments effectively reduce antigenicity while creating free volume spaces upon which the host's native cells can proliferate [37].

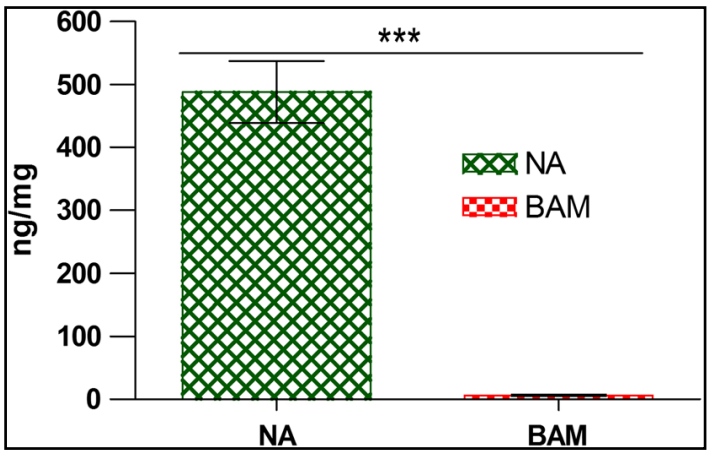

Figure 3. DNA content (ng/mg of tissue) (mean ± SE) in native aorta (NA) and buffalo aortic matrix (BAM). *** P<0.001 versus other [35].

2.1. Preclinical evaluation

Biocompatibility of native, acellular, 1,4-butanediol diglycidylether and 1-ethyl-3-(3-dimethyl aminopropyl carbodiimide (EDC) cross-linked acellular aortic matrix scaffold of bubaline origin was evaluated following subcutaneous implantation in Guinea pigs [34,38,39]. The biocompatibility was evaluated based on gross and histopathological observations and immune responses elicited by the implanted scaffolds. Grossly, no abnormal cellular reaction was observed at the host-scaffold junction in any of the implanted animals. Histopathological observations revealed that the inflammatory response was mild during first 15 days post-implantation and increased at 30 days post-implantation in acellular and cross-linked scaffolds. By day 60, marked in growth of host tissue was observed in EDC cross-linked acellular aortic matrix scaffold. The ELISA and lymphocyte proliferation assay revealed that animals implanted with EDC grafts showed least immune response when compared to others. Therefore, it was concluded that EDC cross-linked acellular aortic grafts were more compatible and had better handling qualities than the other cross-linked grafts [34,38,39]. Native aorta of buffalo origin was decellularized using 1% ionic biological detergent for 48h. The acellular aortic matrix was cross-linked with Glutaraldehyde (GA), 1,4-butanediol diglycidylether (BDDGE) and 1-ethyl-3-(3-dimethyl aminopropyl carbodiimide) EDC for 12 h, 24 h, 48 h and 72 h time intervals. *In vitro* biocompatibility of acellular aortic matrix was determined. Uncross-linked acellular aortic matrix graft was used as control. *In vitro* studies included enzymatic degradation, moisture content percentage, free amino group determination, fixation index, molecular weight analysis and free protein content estimation. Acellular aortic matrix graft cross-linked with 0.6% GA for 48 h and 72 h, 1% EDC and BDDGE for 24 h were found promising [40].

2.2. Clinical evaluation

The decellularized aortic matrix scaffold of bubaline origin was used for repair of congenital umbilical hernias (average size, 6.89 cm in diameter) in nine crossbred Murrah buffaloes (average age, 7.44 months) [41]. After repositioning of the abdominal viscera, an appropriately sized decellularized aortic matrix scaffold with preplaced horizontal mattress natural non-absorbable sutures was introduced underneath the hernial ring. All the animals had an uneventful recovery without clinical signs of wound dehiscence, infection, and recurrence during 3-month follow-up period**.** Results indicated safe use of allogenic decellularized aortic matrix scaffold in buffaloes for the repair of abdominal hernias with adequate strength and minimal foreign body reaction [41].

The umbilical hernias were repaired in nine client-owned cow calves using bubaline derived acellular aortic matrix (AAM). The calves were female and either crossbred Holstein-Friesian (n=7) or undefined breed (n=2). Physical examination in each case revealed a painless, reducible soft swelling with a discernible ring at the umbilicus diagnosed as congenital umbilical hernia. The aortic matrix was prepared as per method described by Devarathnam *et al* [33]. The acellularity of the prepared matrix was confirmed microscopically using haematoxylin and eosin staining. To assess the integrity of the repair,

clinical evaluation of calves was performed at 4-weekly intervals up to 6 months. The technique of hernioplasty using AAM graft appears to be a satisfactory treatment regimen for umbilical hernia repair in calves. The use of non-absorbable, synthetic mesh material has been reported to cause complications such as mesh extrusion, bowel adherence, fistula formation, wound infection, skin erosion and seroma development. To overcome the disadvantages of synthetic meshes, biomaterials may be preferable for the surgical repair of hernias.

In another study successful umbilical hernia repair was done in four crossbred Holstein-Friesian calves with an average age of 7 months and average weight of 60 kg. Average hernial ring diameter was 8 cm. Acellular matrix from buffalo aorta was prepared as per Devarathnam [34]. After proper anaesthesia an elliptical incision was made over the hernial sac and fascia and muscles were separated from the hernial ring. All animals were having slight to strong adhesions. The hernial contents were pushed back in cases where no adhesions or slight adhesions were present. The adhesions were removed by blunt dissection. The hernial ring was freed and repaired by acellular aortic graft using inlay technique The graft was anchored in position by black braided silk No. 2 using horizontal mattress sutures in all the animals. Finally, the skin was closed by black braided silk No.2. All the animals recovered completely and no complication of wound healing was observed up to 10-14th postoperative days [42].

The decellularized aortic matrix scaffold of bubaline origin was used for tracheal segment replacement in a 5-year-old crossbred Holstein-Friesian cow with post anastomotic tracheal stenosis [43]. Under xylazine sedation and local analgesia, through midline cervical incision, the stenotic tracheal segment was exposed and resected. Defect was repaired with the xenogenic decellularized aortic matrix scaffold of bubaline origin, supported by a plastic stent to prevent airway collapse. Postoperative complications were not observed in this animal during a 2.5-year available follow-up [43].

Decellularized aortic matrix of bubaline origin was used for repair of abdominal hernia in six cattle clinically affected with abdominal hernias having mean body weight of 132.50 ± 21.86 kg and mean hernia size of 40.55 ± 15.42 cm^2. The aortic matrix was prepared as per method described by Devarathnam *et al* [33]. Clinical, hematobiochemical and antioxidant parameters were evaluated to assess biocompatibility of the xenogenic AAM. Cattle with the decellularized aortic matrix implant recovered uneventfully and remained sound. Hemato-biochemical and antioxidant findings were unremarkable. The xenogenic bubaline aortic matrix shows excellent repair efficiency and biocompatibility for abdominal hernia repair in cattle without complications [35].

The decellularized aortic matrix scaffolds of bubaline origin crosslinked with 1-ethyl-3-3-dimethylaminopropyl carbodiimide hydrochloride (EDC) were used for the repair of inguinal hernias in five horses [44]. The matrices were applied using an inlay technique. All animals that underwent hernia repair demonstrated successful healing without clinical signs of wound dehiscence, infection, or recurrence during a 6-month available follow-up. The EDC-crosslinked decellularized aortic matrix could be used safely in horses for the repair of the inguinal hernia with adequate strength and minimal foreign body reaction [44].

In goats, the decellularized aortic matrix scaffolds of bubaline origin prepared as per method described by Devrathnanam *et al* [33] was successfully used for the repair of large ventral hernia (ring size, 12 cm in diameter) in a 35-kg 3-year-old Jamuna Pari buck. The buck recovered completely without clinical signs of wound dehiscence or infection. Ventral hernia repaired with decellularized aortic matrix result in no herniation up to 3-month after reconstruction [45].

3. Preparation of acellular diaphragmatic matrix scaffold

Fresh diaphragm (tendinous portion) of buffalo was collected from a local abattoir in chilled (4 °C) sterile phosphate buffered saline (PBS, pH 7.4), containing 0.048% gentamicin, 0.0205% EDTA and 0.1% sodium azide, and processed immediately. After initial washing, diaphragm tissue was subjected to 0.5%, 1%, 2%, 3% and 4% sodium dodecyl sulfate (SDS) solution, respectively. The tissue was continuously agitated at the rate of 250 rpm in SDS solutions on magnetic stirrer for 12 h, 24 h, 48 h, and 72 h at room temperature. They were evaluated based on gross, histological, scanning electron microscopic observations, DNA contents evaluation, fourier transform infrared spectroscopy, 4,6-Diamino-2-phenylindole dihydrochloride staining, agarose gel electrophoresis and *in-vitro* study of matrix metalloproteinases. Treatment of a diaphragm with 2% sodium dodecyl sulfate solution for 48h revealed orderly arranged collagen fibers. Prepared buffalo diaphragm matrix was extensively rinsed with sterile PBS to remove residual detergent and stored in sterile PBS containing 0.048% gentamicin at -20 °C till clinical use. Sterility test of stored sterile PBS was performed to check any bacterial or fungal growth. The prepared matrices were later tested in clinical cases of abdominal wall defects of different species of animals.

The prepared acellular diaphragm scaffold of bubaline origin grossly, showed good tensile strength and elasticity. Histologically, native diaphragm showed cell nuclei and acellular diaphragm showing no nuclei after H&E and Masson's trichrome staining (Figure 4a,b). Treatment of a diaphragm with 2% SDS solution for 48 h revealed orderly arranged collagen fibers within bovine diaphragm matrix (BDM). Treatment of a diaphragm with 3% and 4% SDS solutions at all incubation period resulted in extensive damage to collagen fibers with increased porosity. The SEM examination of the native diaphragm revealed compact collagen fibers with low porosity. Thickness of collagen fibers varied between 2.074 µm and 4.818 µm. The BDM showed intact collagen structure and integrity. Thickness of collagen fibers was slightly lesser as compared to the native diaphragm, and varied between 1.454 µm and 4.154 µm. Further, SEM examination also confirmed effective decellularization of the bubaline diaphragm and preservation of collagen structure and integrity within BDM (Figure 5a–c). Extracts from decellularized diaphragm tissue show considerable removal of DNA material, with absence of DNA band. This is indicative of effective removal of remnant DNA from developed BDM. The 4,6-Diamino-2-phenylindole dihydrochloride (DAPI) stained sections showed resulted in loss of cellularity without affecting the three-dimensional structure of collagen fibers. Fresh diaphragm from deceased donor buffalo was decellularized using 2% sodium deoxycholate solution for 48 h [46].

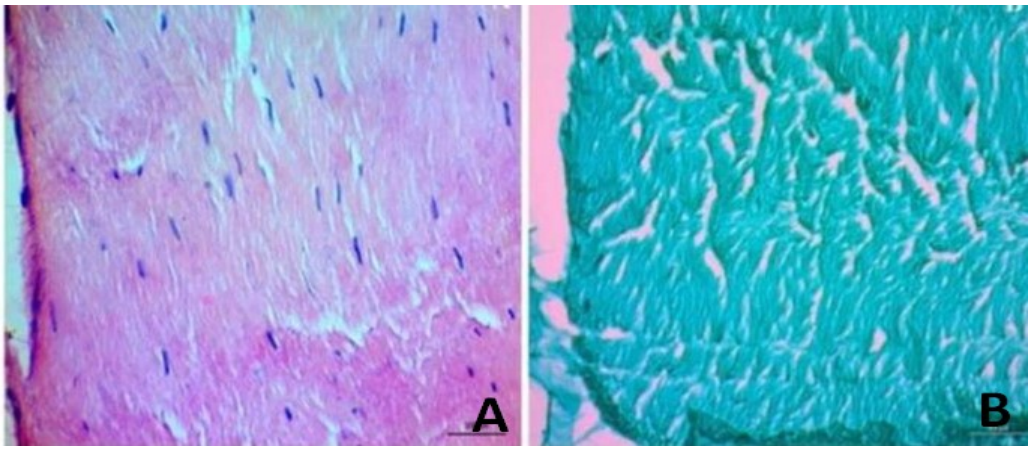

Figure 4. (a) Microscopic images of native buffalo diaphragm after hematoxylin-eosin staining; **(b)** Masson's trichrome staining (X 40; scale bar 200 µm).

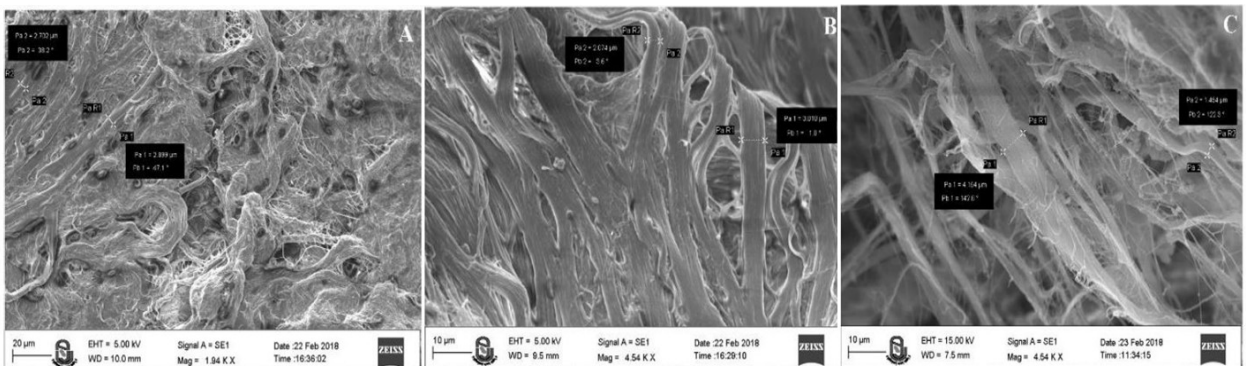

Figure 5. (a)-(b) SEM images of native diaphragm (ND); **(c)** acellular buffalo diaphragm matrix (BDM) [46].

The increasing use of bio scaffolds for tissue repair has prompted its development. They are typically prepared after decellularization of source tissues. Carpo *et al* [47] have suggested that no visible nuclei per histologic evaluation via eosin and hematoxylin is a part of the minimum criteria for effective decellularization in terms of the DNA content remaining in decellularized biological scaffolds. In the present study, xenogenic BDM scaffold was developed from fresh diaphragm of buffalo origin using aqueous SDS solution. SDS was used in the present study because it is readily available, cost effective and has been used for decellularization of other biological tissues such as aorta [46] and skin [48,49]. Histologically, treatment of fresh diaphragm with 2% SDS solution for 48h resulted in complete loss of cell nuclei, and retention of the distinctive, natural, three-dimensional structures of the collagen within developed matrix. The SDS an ionic detergent, is effective for solubilizing both cytoplasmic and nuclear cellular membranes [50]. It is typically more effective for removing cell residues from tissue compared to other detergents [51]. Host tissue response to subcutaneously implanted diaphragm matrices was studied. Extent of host reaction was more in the native scaffolds as compared to acellular scaffolds [52]. A recent method to assess effective decellularization of xenogenic tissue is by quantification of remnant DNA [53]. DNA retained in decellularized tissues may induce an immune response

and foreign body reaction by the host to scaffold implants. Carpo *et al* [47] have suggested that 50 ng/mg dry weight is a part of the minimum criteria for effective decellularization in terms of the DNA content remaining in decellularized biological matrices. This value was approached with in the present study, DNA content was significantly ($P<0.045$) lesser in BDM as compared to the native diaphragm. Treatment with 2% SDS solution for 48 h resulted in almost complete reduction in DNA content, indicating effective acellularity. Absence of DNA band on Ethidium Bromide-stained agarose gel further indicating loss of DNA from the prepared matrix.

3.1. Preclinical evaluation

Healing potential on full thickness skin wounds of primary mouse embryonic fibroblasts (p-MEF) seeded on bubaline acellular diaphragm matrix (ADM) was studied. A 20 × 20 mm^2 sized full thickness skin defect was created on dorsum of forty-eight Sprague Dawley rats and randomly divided into four equal groups. The defect in group I (control) was left open whereas, groups II, III and IV were covered with autograft, ADM and p-MEF seeded ADM, respectively. Wound healing was evaluated based on gross, immunologic, and histopathologic observations. Gross healing observation with p-MEF seeded ADM group showed significantly ($P<0.05$) higher wound contraction as compared to others. There was higher B-cell response in animals with ADM and p-MEF seeded ADM implants. The p-MEF seeded ADM implantation caused markedly fast wound closure with well-formed granulation tissue dominated by fibroblast proliferation, collagen deposition, and complete early regenerated epithelial layer. It was found that p-MEF seeded ADM has healing potential and shown better healing of full thickness skin wounds in rats [54].

Remya *et al* [55] investigated the potential of mesenchymal stem cells tailored acellular bubaline diaphragm for the repair of full-thickness abdominal wall defects in a rabbit model. Tissues obtained from bubaline diaphragm were decellularized and bioengineered by seeding with rabbit bone marrow derived mesenchymal stem cells (r-BMSC). Full-thickness abdominal wall defects of 3 × 4 cm^2 size were created in a rabbit model and repaired. Seeding with r-BMSC significantly increased ($P<0.05$) the collagen deposition and biomechanical strength of the scaffold. The bioengineered r-BMSC seeded acellular bubaline diaphragm showed superior biomechanical strength. Tailoring of the scaffolds with the r-BMSC also resulted in significant reduction ($P<0.01$) in antibody and cell mediated immune reaction [55].

3.2. Clinical evaluation

Acellular diaphragm matrix (ADiaM) of bubaline origin were used to reconstruct the abdominal wall defects. The ADiaM matrices were applied in eight clinical cases of abdominal wall defects of different species of animals. Animals with ADiaM matrices recovered uneventfully and remained sound at least up to 3 months. Hematological and immunological findings were unremarkable. Bubaline diaphragm showed excellent repair efficiency and biocompatibility for abdominal wall defects repair in animals without complications [56]. In another study prepared ADiaM matrices were used for repair of

abdominal wall defects in four different species of animals. The abdominal wall defects repaired with ADM remained sound over a period of 3 months. All the defects repaired with ADM healed completely without graft rejections. The present study suggested that ADM may be used safely for repair of abdominal wall defects in different species (buffalo, cattle, goat, and pig) of animals [57]. The buffalo diaphragm after decellularization with 2% sodium deoxycholate for 48 h was used for umbilical hernioplasty in 12 crossbred Landrace pigs. Treatment with 2% sodium deoxycholate leads to complete decellularization at 48 h. All the hernias repaired with acellular diaphragm matrix healed completely without graft rejection and remained sound over a period of 3 months [46].

Acellular diaphragm matrix of bubaline origin was successfully used in a pig and a dog for the repair of umbilical and perineal hernia, respectively. The reconstructive surgery was done using acellular diaphragm matrix scaffold. Post-operatively both animals showed successful augmentation of reconstructive procedures with this newer approach. Remya *et al* [58] performed reconstruction of umbilical hernia with acellular diaphragm matrix in a 7-month-old crossbred calf using inlay technique. The animal completely recovered after 30 days without postoperative complications. Perineal hernioplasty was performed in a male spitz dog using acellular diaphragm matrix of bubaline origin. The animal had an uneventful recovery [59]. Hysterocele in a Dachshund dog was surgically corrected with decellularised diaphragm matrix of bubaline origin [60]. A large inguinal hernia in a horse was successfully repaired using acellular diaphragm matrix [61]. Acellular diaphragm matrix of bubaline origin was found to be a promising and immuno-tolerable prosthetic for hernioplasty [62]. Acellular diaphragm matrix was also used for the repair of umbilical hernia in two cow calves [63].

4. Preparation of acellular ruminal matrix scaffold

Fresh rumen tissue of water buffalo (*Bubalus bubalis*) was procured from the local abattoir and immediately preserved in chilled 1x phosphate buffer saline (PBS), (pH 7.4) solution containing 0.1% amikacin and 0.02% EDTA and was made acellular. Decellularization protocols were optimized based on the principle of maximum removal of cellular contents with minimum damage to basic tissue architecture [64,65].

Macroscopic observations showed that the keratinized mucosal layer was easily scrubbed off and serosal layer was separated with slight mechanical assistance. The isolated delaminated and decellularized bubaline rumen was kept in 70% ethanol for sterilization. The bovine rumen tissue matrix after treatment with biological detergents appeared soft, white, and slightly spongy in consistency then native tissue.

Microscopically, native bovine rumen showed keratinized epithelium on mucosal surface. Lamina propria is the luminal portion of the propria-submucosa, which includes a dense layer of extracellular matrix and serosal layer. The delaminated propria-submucosal layer showed cellularity, tunica muscularis and thick collagen fibers. Native bovine rumen showed dense compact arrangements of collagen fibers. The delaminated bovine rumen matrix subjected to ionic, non-ionic, zwitterionic biological detergents and enzyme treatment at 0.5% and 1% conc. for different time intervals. All the specimens treated for different time

intervals were adequately decellularized, except enzyme treatment. Treatment with trypsin enzyme was inefficient in decellularization during various time intervals. Complete acellularity was achieved with 1% sodium deoxycholate at 72 h [65]. Masson's trichrome staining revealed thick, transversely, and longitudinally arranged collagen fibers. The quantification was a direct measure to confirm the effectiveness of the processes, since the DNA is present in the active nucleus of cell. In the native rumen matrix, abundant cell components and nucleic acids were present. However, after the decellularization, cells and nucleic acids were hardly observed in ECM. The DNA concentration in native bovine rumen matrix was 48.09 ηg/µl. After decellularization by different protocols, the DNA conc. decreased and ranged from 12.27 ± 0.07 ηg/µl to 38.17 ± 0.05 ηg/µl in different detergents treatment groups [64,65]. Collagen based biomaterials from buffalo ruminal wall were developed using sodium deoxycholate detergent for tissue engineering [66].

4.1. Preclinical evaluation

The efficacy of acellular rumen matrix and matrix seeded with r-BMSCs for the repair of full thickness skin wounds in diabetic rats was tested. The diabetes in rats was induced by intra-peritoneal (i/p) administration of Streptozotocin (60 mg/kg body weight). The rats with blood sugar level more than 280 mg/dl were considered diabetic. The rat bone marrow mesenchymal stem cells were isolated as per as per standard protocols. After 3rd passage, the stem cells were detached with 0.25% trypsin-EDTA solution and then were seeded on acellular rumen matrices to prepare 3-D bioengineered scaffolds from bubaline rumen.

Forty-five rats of either sex was randomly divided into 3 groups having 15 rats in each group. After surgical anesthesia, a full thickness 20 × 20 mm^2 skin defect was created on dorsal thoracic area in all the rats. The defects of groups II and III were repaired immediately with acellular rumen matrix and matrix seeded with r-BMSCs. Animals of group I were treated as control and where wound was left open [64,65].

A significant decrease ($P<0.05$) in wound area was recorded in all the groups up to day 28, except in group III where complete healing was observed on day 28. Complete healing was recorded on day 42 in other groups. A significant increase ($P<0.05$) in percent contraction was observed in all the groups during the observation period. Computerized planimetry showed that on day 14, the acellular rumen matrix was firmly adhered with the underlying pinkish granulation tissue and on day 42, the wound healed up completely with no scar. However, in group III where rumen matrix was seeded with r-BMSCs the wound healed up completely on day 28. In control group on day 42 the wound healed completely by severe contraction leaving a large scar. Based on the study, it can be concluded that acellular rumen matrix developed from buffalo rumen was found biocompatible and biodegradable and acellular rumen matrices seeded with r-BMSCs mimic the properties of skin and be considered as novel biomaterials for the repair of full thickness diabetic dermal wounds [64,65].

4.2. Clinical evaluation

A total of 26 buffalo calves aged between 3 and 27 months and weighing 50–115 kg were treated with acellular rumen matrix of buffalo. The animals were having umbilical hernia with an average diameter of hernial ring being 6–12.5 cm. Physical examination revealed painless, reducible soft swelling at the umbilicus. Multiple loops of bowel could be palpated traversing the hernial ring. At the time of presentation, the animals have normal temperature, respiration, and pulse rate [67]. The calves were sedated using xylazine HCl @ 0.1 mg/kg body weight and circular infiltration at the site using 2% lidocaine HCl. The animals were controlled in lateral or dorsal recumbency as per the convenience. The rumen acellular matrix of buffalo origin was prepared as per Singh *et al* [65]. Matrix was prepared according to the size of the hernial ring. An elliptical skin incision was given to expose the hernial sac which spanned the length of the hernia and extended 2 cm beyond the cranial and caudal margins of the hernial ring. Underlay technique was used for the implantation of buffalo rumen acellular matrix. The subcutaneous tissues were closed using number 2–0 polyglactin 910 placed in a simple continuous suture pattern. The skin incision was then closed with silk sutures in a horizontal mattress suture pattern. Postoperatively, the animals were administered with amoxicillin-cloxacillin antibiotic combination by intramuscular route for 5 days and analgesic meloxicam (0.5 mg/Kg) for 3 days. The skin wounds were dressed routinely with 0.1% povidone iodine. Skin sutures were removed on postoperative day 12. The operated animals were followed either by regular visits or calling the owner during one year post surgery [67].

At the end of the first post-implantation week, a thick and slightly hard mass resembling the size of implanted matrix was palpated at the site of the hernioplasty. The thick and hard mass of tissue at the site of implantation was palpable until the end of the second post-implantation week. These masses subsided by the end of the third post-implantation week. After the fifth post-implantation week the masses become thinner and fibrous, and were difficult to feel on palpation. The umbilical region looked normal on inspection. All the animals recovered uneventfully. Clinical signs like wound dehiscence, infection, or hernial recurrence were not recorded in any of the cases. All the animals were observed up to 12 months after hernioplasty. After decellularization rumen matrices were used for umbilical hernioplasty in buffalo calves and the results demonstrated the uncomplicated healing of the repaired area, without hernial recurrence or rejection [67].

5. Preparation of acellular dermal matrix scaffold

Buffalo skin is characterized by dermal papillae enclosing papillomatous epidermis. In buffaloes, the average thickness of the skin, main epidermis, papillomatous epidermis, and cornium is 6.5 mm, 50 mm, 115 mm, and 11 μm, respectively. The average number of hair follicles per cm^2 of skin is 394. Each hair follicle is accompanied by two large lobulated sebaceous glands in the buffalo [68]. Fresh ventral abdominal skin of buffalo was procured from the local abattoir. The skin was preserved in cold physiological saline just after the collection and later on rinsed with normal saline to remove the adhered blood. The maximum

time between the retrieval and the initiation of protocols was less than 4 h. The skin samples were cut into 2 × 2 cm^2 size pieces. This phase was divided into two parts. Firstly, the protocol for de-epithelialization was optimized and then protocol to prepare acellular dermal matrix was optimized [69].

For de-epithelialization trypsin and sodium chloride was used. Based on the gross observations the desirable removal of epidermis in a single layer was not possible with trypsin and NaCl solutions. The separation of the epidermis was done with the help of dermatome. Histological observations also showed that removal of epidermis was not possible with trypsin and NaCl solutions. Removal of epidermis with dermatome was achieved. The de-epithelialized skin of buffalo was subjected to 2% trypsin for 24 h. Then 2%, 3%, 4% and 5% SD solution was used for 12 h. Then the samples were again treated with 2% trypsin for 12 h. The observations were recorded from upto 108 h of post treatment. The samples were continuously agitated on shaker. In next step the samples were subjected to same time and concentration of detergents treatment. The samples were washed thrice at every step to rinse the residuals of previous steps. The samples were subjected to SDS, tritron-x100 and tween-20 in the concentrations described above. All the solutions were filter sterilized and each procedure was performed aseptically. The time intervals, concentration and pH of the chemicals were modified to obtain the desired result [69].

Treatment with SD and SDS in 2% and 3% showed an 80% to 90% decrease in the cellular contents. The collagen fibers were mildly thin in 2%, became mildly thick and loose in 3% solution. In 2% triton X-100 detergent the scaffolds showed 80% to 90% decrease in the cellular contents and in 3% solution scaffolds showed 90% to 100% decrease in the cellular contents. The collagen fibres were mildly thick and mildly loose in architecture. In 2% tween-20 biological detergent the scaffold showed 80% to 90% decrease in the cellular contents and 90% to 100% decrease in 3% solution. Mildly thick and moderately loose collagen fibers were seen in both the concentration. In 4% and 5% concentration of SD the scaffolds showed 90% to 100% decrease in the cellular contents. The collagen fibres were mildly thick and mildly loose in 4% concentration and became moderately loose in 5% concentration. In 4% and 5% concentration of SD the scaffolds also showed 90% to 100% decrease in the cellular contents. Collagen fibers were mildly thin and loose in 4% and mildly thick and moderately loose in 5% concentration. In both the concentration triton X-100 and tween-20 scaffolds showed 90% to 100% decrease in the cellular contents with mildly thick collagen fibers. The collagen fibers were mildly loose in 4% and moderately loose in 5% concentration solution [69].

Decellularization techniques resulted in the removal of nucleus and cytoplasmic cellular components, lipids, and its membranes along with soluble proteins and basement membrane components of cellular material. We tried to optimize protocols for de-epithelialization of buffalo skin using trypsin and sodium chloride. However, the skin of the buffalo was not completely de-epithelialized by the trypsin and sodium chloride treatment. The epidermis of buffalo skin was separated using dermatome. Trypsin attacks the desmosome complex between the cells thus remove the epidermis in a single layer [70], but on increased time intervals the partially digested epidermis came out due to partial digestion of the cells.

However, NaCl was not found suitable to separate the epidermis in this species which was effective in human skin.

The anionic biological detergent gives better results than non-ionic biological detergents. Sodium dodecyl sulfate was found very effective for the removal of cellular components from tissue. Compared to other detergents SDS yields complete removal of nuclear remnants and cytoplasmic proteins. Ionic detergents are effective for solubilizing both cytoplasmic and nuclear cellular membranes, but tend to denature proteins by disrupting protein-protein interactions [50]. In general, anionic detergents are used extensively in decellularization protocols due to their mild effects on tissue structure. Non-ionic biological detergents (TritronX-100) do not usually denature proteins. They are suited to investigate the subunits structure of membrane proteins. It inhibits protein-protein and protein-lipid bonding. The allogenic dermis is antigenic because donor cellular components evoke an immune reaction following transplantation [71].

5.1. Clinical evaluation

Acellular dermal matrix scaffolds prepared from buffalo skin was successfully used for the reconstruction of umbilical/ventral hernias in four buffalo calves. The animals were presented with the history of swelling over the umbilical region. All these cases were diagnosed as congenital umbilical hernias. In one buffalo calf, the swelling was present just behind the 10th rib and the swelling was developed due to gore injury. On clinical examination, the costal cartilages were fractured but the diaphragm was intact. This case was diagnosed as ventral hernia [72]. In all the cases acellular dermal matrix prepared using SD biological detergent [69].

The operation was performed in dorsal recumbency for treating the umbilical hernia and in lateral recumbency for ventral hernia. After aseptic preparation of the site, Xylazine (0.1 mg/kg intramuscularly) was administered and 2% lignocaine hydrochloride was infiltrated at the site in all the animals, except in case of ventral hernia where intercostals nerve block was also performed. After proper analgesia an elliptical incision was made over the hernia sac and fascia and muscles were separated from the hernia ring. The hernial contents were pushed back into the abdominal cavity. The adhesions were removed by blunt dissection. The adhesions were very strong in the case of ventral hernia. The hernial ring was separated and repaired by acellular graft as mentioned above using the inlay technique. The grafts were anchored in position by black braided silk No. 2 using horizontal mattress sutures in all the animals having an umbilical hernia. In the case of ventral hernia, the 10th rib was clearly visible after reposition of the abdominal organs. No soft tissue was present anteriorly to support the graft. The graft was anchored anteriorly on the intercostals muscles of the 9^{th} intercostals space. The subcutaneous tissue was repaired using catgut No. 2. Finally, the skin was closed by black braided silk No. 2. Post-operative treatment included intramuscular injection of Enrofloxacin (5 mg/kg) for 7 days and Meloxicam (0.2 mg/kg) for 3 days. Daily dressing of the suture line was performed with 5% Povidone iodine. The owner was advised to give the concentrate diet. The skin sutures were removed on 10–14th postoperative days as per wound healing status. To evaluate the long term results a telephonic survey was carried

out. All the animals recovered completely and no complication of wound healing was observed up to 10–14 post operative days. The present study revealed that acellular dermal grafts of buffalo origin proved to be a good biocompatible biomaterial for the reconstruction of hernia. It is easy to prepare and can be clinically practiced in other species of animals also [72]. Acellular dermal grafts have been used for the repair of abdominal wall defects in experimental animals with excellent results [73,74].

6. Preparation of acellular reticulum extracellular matrix

The ECM scaffolds can be derived from the reticulum of the forestomach. Such ECM scaffolds contain the lamina propria and submucosa (propria-submucosa) layers of the forestomach wall. Bovine reticular matrix is selected for this study as histology shows that the lamina propria is unusually dense, whereas the abluminal side of the FM scaffold is structured as an open reticular matrix. These differences serve an important role in epithelial regeneration, as the dense side acts as a barrier to cell migration, while the less dense side does not present a barrier and therefore allows cell invasion. This structure makes the FM well suited for encouraging epithelial regeneration on the dense luminal side of the matrix, and fibroblast invasion on the less dense abluminal side of the matrix, when used as a medical device for tissue regeneration. In contrast, submucosal tissue grafts derived from the glandular stomach and the urinary bladder has a uniform density. The dense layer of ECM from the lamina propria contributes to the increased thickness and strength of FM scaffolds compared to those derived from other organs. The large surface area of the forestomach and the increased thickness and strength of scaffolds derived from the forestomach allows the isolation of larger ECM scaffolds from the forestomach than is possible from other organs [75].

Reticulum of buffalo was procured from the local abattoir. Immediately, after collection the reticulum was be kept in cold physiological saline solution containing 0.02% EDTA and antibiotic (Amikacin @ 1 mg/ml). Tissue was cut from sides to obtain a flat sheet and was processed. Treatment with detergent was done up to 72 h and the solution was changed at every 12 h time intervals and samples were collected at 12 h, 24 h, 48 h and 72 h time intervals for histopathological, scanning electron microscopic evaluation and DNA quantification. The bovine reticulum tissue treated with different biological detergents and enzymes at 0.5% concentration appeared soft, whiter, and slightly spongy in consistency than native tissue at different time intervals.

Microscopically, native bovine reticulum showed keratinized epithelium on mucosal surface. Lamina propria is the luminal portion of the propria-submucosa, which includes a dense layer of extracellular matrix and serosal layer. Microscopic examination also revealed complete removal of keratinized epithelium and serosal layer. The delaminated propria-submucosa, layer showed cellularity, tunica muscularis and thick collagen fibers. Native bovine reticulum showed dense compact arrangement of collagen fibers clearly visible in Masson's trichrome staining (Figure 6a–c).

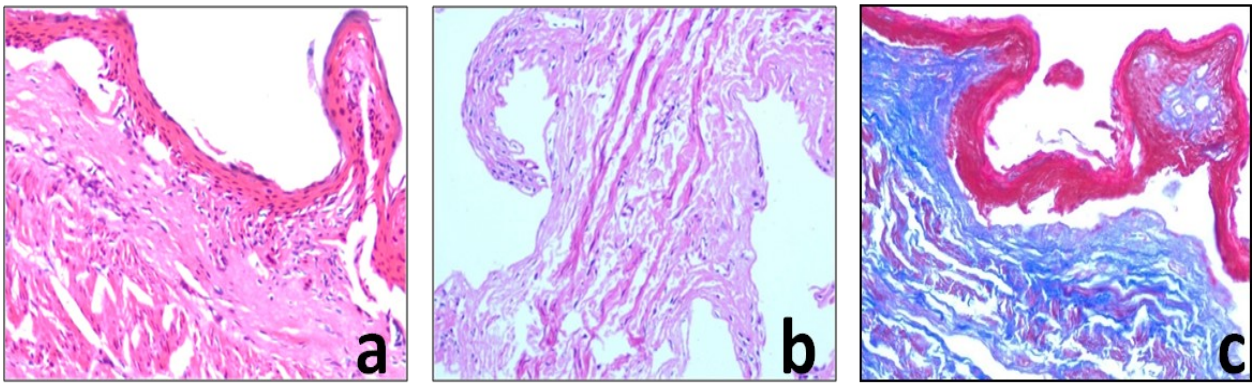

Figure 6. (a) Native bovine reticulum showing keratinized epithelium on mucosal surface); **(b)** delaminated propria-submucosa, showing cellularity, tunica muscularis and thick collagen fibers) (H and E stain, X-100); **(c)** native bovine reticulum showing clearly dense compact arrangement of collagen fibers (Masson's trichrome stain, x-100).

The delaminated reticulum treated with 0.5% SD for 12 h under constant agitation showed 85% to 90% loss of cellularity along with presence of cellular debris. Treatment for 24 h showed slight increase in loss of cellularity *i.e.*, more than 90% and cellular debris was noted. At 48 h time interval sub mucosal layer was completely acellular. The collagen fibers were compact with moderate porosity than the native tissue. No cellular debris was observed between the spaces of thick collagen fibers. At 72 h time interval rECM showed thin, heavily loose arranged collagen fibers with very high porosity. No cellular debris was evident. Treatment with 0.5% Triton-x 100 for 12 h under constant agitation showed 50% to 60% loss of cellularity with high cellular content, thick and compact collagen fiber arrangement with least porosity. At 24 h no further loss of cellularity was observed. High cellular contents, thick and compact collagen fiber arrangement with least porosity was observed. At 48 h time interval loss of cellularity increased up to 80% with moderate cellular debris and porosity. At 72 h time interval no further increase in cellularity and porosity was observed. Mild cellular debris and damage in collagen fiber arrangement was observed at this stage (Figure 7a–d).

Treatment with 0.5% tri (n-butyl) phosphate for 12 h and 24 h showed no effect on loss of cellularity. High cellular contents were noted at 12 h as compared to 24 h. At 48 h nearly 80% of decellularization was observed. At 72 h more than 80% decellularization and loss of cellular debris was seen. Treatment with trypsin enzyme was inefficient in decellularization during various time intervals. No loss in cellular content with any effect on collagen fibre arrangement was observed at different time intervals. All the specimens treated for 12 h, 24 h, 48 h and 72 h were adequately decellularized, except enzyme treatment. However, the best scaffold was prepared by treatment with 0.5% SD. On Masson's trichrome staining thick, transversely, and longitudinally arranged collagen fibers were observed.

Scanning electron microscopic observations revealed loss of cellularity. The native bovine reticulum matrix consisted of dense and closely packed collagen fibers. Delaminated reticulum showed uneven luminal surface and aggregated collagen bundles. Ultra structure information revealed the events occurred during decellularization of delaminated bovine reticulum. The fine filamentous non-collagenous content of the reticular ECM was also clear,

forming a fine weave between the collagen bundles. Within the matrix detailed collagen microarchitecture was evident containing numerous interlacing fibres of various thickness, pores, and open channels [76]. The DNA concentration in native bovine reticulum was 47.08 ± 0.02 ηg/µl. After decellularization by different protocols, the DNA concentration decreased and ranged from 11.23 ± 0.06 ηg/µl to 37.13 ± 0.04 ηg/µl. Significant decrease ($P<0.01$) in DNA contents showed the effectiveness of treatment for decellularization. Treatment with SD showed effective removal of cells. Quantification of residual DNA in animal derived biological scaffold materials is one of technical specifications for evaluating decellularization process and immunogenicity risk [77]. Treatment with 0.5% SD for 48 h resulted in completely acellualr matrix as evaluated by histology, scanning electron microscopy and DNA quantification. Treatment with enzyme does not show any significant reduction in cellularity.

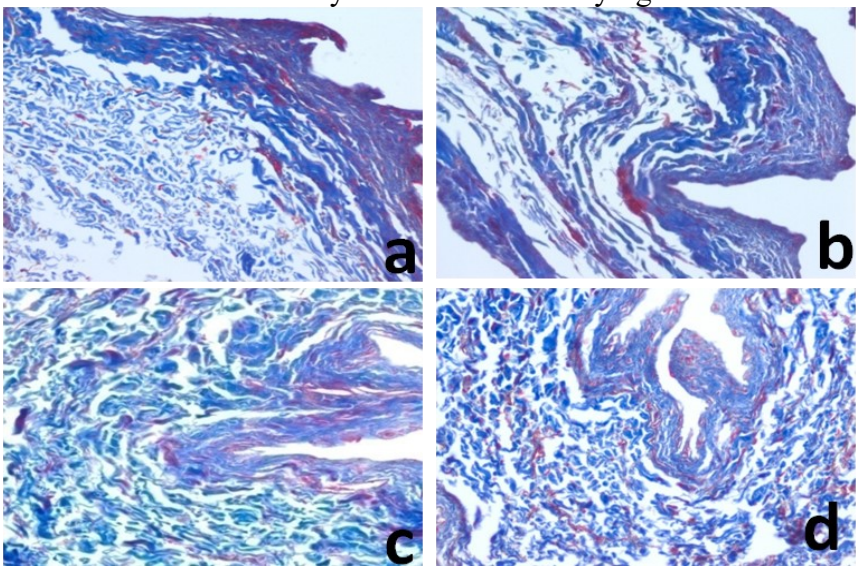

Figure 7. Delaminated reticulum at 72 h with different treatments, **(a)** sodium deoxycholate; **(b)** tritron x-100; **(c)** tri (n-butyl) phosphate; **(d)** trypsin (Masson's trichrome staining, X-100).

6.1. Preclinical evaluation

Thirty-six clinically healthy adult Wistar rats of either sex were used in the study and randomly divided into three groups having twelve animals in each group. Under general anesthesia, a full-thickness skin wound (20×20 mm^2) was created on the dorsum of each rat. The defect in group I was kept as open wound and was taken as control. In group II, the defect was repaired with commercially available collagen sheet (b-CS). In group III, the defect was repaired with reticulum derived extracellular matrix of bovine origin (b-REM). All the wounds were dressed with standard dressing material. The wounds were photographed on days 0, 3, 7, 14, 21 and 28 post-surgery and evaluated by independent observer to assess the quality of wound healing and cosmetic outcome [78].

Healing was evaluated on the basis of wound contraction, gross, haematological, immunological and histopathological observations. The surgical wounds appeared apparently healthy in all the animals of different groups throughout the period of observation. None of the wound showed any complication like infection or pus formation. A significant decrease

($P<0.05$) in wound area was recorded in all groups on day 28. Significant increase ($P<0.05$) in percent contraction was observed in all groups during observation period. The humoral response in rats elicited by applying of different types of materials was determined by indirect ELISA. In all the treatment groups there were relative rise in antibody titer from day 0, 21 to day 28 post-implantation. In histopathological observations biopsy samples were examined with routine H&E staining and special staining for collagen with Masson's trichrome Stain. In terms of surface epithelium presence and thickness, the defect repaired with reticulum derived extracellular matrix of bovine origin (b-REM) showed early epithelialization with an early proliferation of fibroblast and angiogenesis to fill the defects. This group showed an early resemblance to the normal skin without any contraction than other groups. On critical analysis of the observed parameters, it was found that b-REM has healing potential and shown better healing response than commercially available collagen sheet to reconstruct full thickness skin wounds in rats [78].

7. Preparation of acellular matrix from omasum

Protocols for decellularizing the buffalo omasum were optimized. Omasum of buffalo was procured from the local abattoir. Immediately, after collection the omasum was be kept in cold physiological saline solution containing 0.02% EDTA and antibiotic (Amikacin @ 1 mg/ml). After thorough cleaning of the bovine omasum, de-epithelialization was done in hypertonic solution 2M NaCl for 6 h. The keratinized epithelium of omasum was easily scrubbed off by blunt surface of BP-handle. The serosal layer was separated mechanically with forceps. The omasum after de-epithelialization and delamination was cut into 4×4 cm^2 size pieces and placed in biological detergent and enzymes to carry out decellularization protocols. The omasum was made acellular using ionic detergent (sodium deoxycholate), non-ionic detergent (triton x-100) zwitterionic detergent (tri (n-butyl) phosphate) and enzyme (trypsin) in 0.5% concentration. Treatment with detergents was done up to 72 h and the solution was changed at every 12 h time intervals and samples were collected at 12 h, 24 h, 48 h and 72 h time intervals for gross, microscopic observations, DNA quantification and SDS-PAGE analysis. The prepared acellular omasum matrix was stored in PBS solution containing 1 mg/ml amikacin at 4 ℃ until use [79].

Gross examination at 6h time interval revealed that the keratinized epithelium was easily scrubbed off by blunt edge of BP handle while the serosal layer was delaminated by applying mechanical assistance and results in complete de-epithelialization and delamination.

Microscopic examination revealed complete removal of keratinized epithelium and serosal layer. Native bovine omasum showed keratinized epithelium on mucosal surface. Lamina propria is the luminal portion of the propria-submucosa, which includes a dense layer of extracellular matrix and serosal layer (Figure 8a,b). Delaminated omasum treated with 0.5% SD for 12 h under constant agitation showed 85% to 90% loss of cellularity along with presence of cellular debris. Treatment for 24 h showed slight increase in loss of cellularity *i.e.* more than 90% and cellular debris. At 48 h time interval sub mucosal layer was completely acellular. The collagen fibers were compact with moderate porosity than the

native tissue. No debris was observed between the spaces of thick collagen fibers. At 72 h time interval ECM showed thin, loosely arranged collagen fibres with very high porosity. No cellular debris was evident at this stage (Figure 9a,b) [79].

The treatment with 0.5% triton x-100 for 12 h under constant agitation showed 30% to 40% loss of cellularity with high cellular content, thick and compact cellular fiber arrangement with least porosity. At 24 h no further loss of cellularity was observed. High cellular content, thick and compact collagen fiber arrangement with least porosity was observed. At 48 h time interval loss of cellularity increased up to 60% with moderate cellular debris and porosity. At 72 h time interval, there was no further decrease in cellularity and porosity was observed. Mild cellular debris and damage in collagen fiber arrangement was observed at this stage.

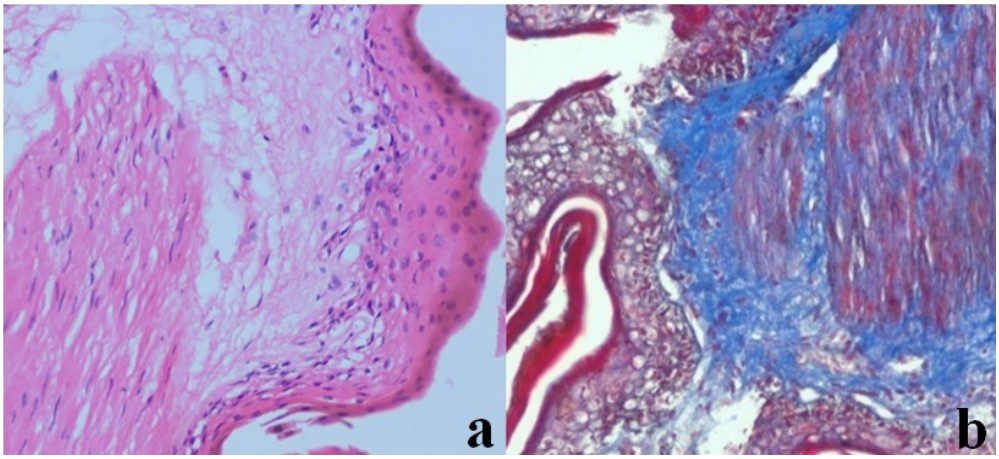

Figure 8. (a) Native bovine omasum after delamination showing cellularity, loose muscular layer, and collagen fibers (H&E stain, x-200), **(b)** Showing dense compact arrangement of collagen fibers (Masson's trichrome staining, x-200) [29].

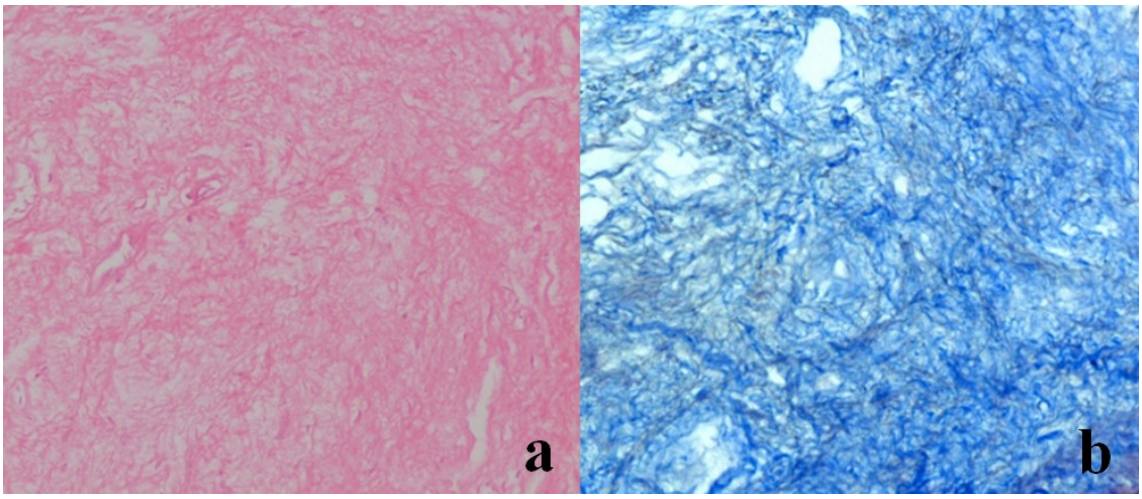

Figure 9. (a) Microscopic photograph showing no cellular debris between the spaces of thick collagen fibers (H&E stain, X200); **(b)** Masson's trichrome staining, x-200 [29].

The treatment with 0.5% tri (n-butyl) phosphate for 12 h and 24 h showed no effect on loss of cellularity. The high cellular contents were noted at these time intervals. At 48 h and 72 h, nearly 80% decellularization with loss of cellular debris and damage in collagen fiber arrangement was observed. Treatment with trypsin (enzyme) was inefficient in decellularization at various time intervals. No loss in cellular content was observed at different time intervals [79].

Microscopically, native bovine omasum showed keratinized epithelium on mucosal surface. Lamina propriais the luminal portion of the propria-submucosa, which includes a dense layer of extracellular matrix and serosal layer. The delaminated propria-submucosa layer showed cellularity, tunica muscularis and thick collagen fibers. The native bovine omasum laminae showed dense compact arrangement of collagen fibers clearly visible in Masson's trichrome staining. Complete acellularity was achieved with 0.5% sodium deoxycholate at 48 h. Masson's trichrome staining revealed thin, transversely, and longitudinally arranged collagen fibers. Microscopic photograph shows no cellular debris between the spaces of thick collagen fibers as observed in H&E staining and in Masson's trichrome stain.

In the native omasum, abundant cell components and nucleic acids were present. However, after the decellularization, cells and nucleic acids were hardly observed in ECM. The DNA concentration in native bovine omasum was 82.40 ng/µl. After decellularization the DNA concentration significantly decreased in SD treated group but no difference was observed in other detergents treated as well as enzyme treated group at 24 h and 48 h time intervals [79].

In the study four different protocols were used to obtain the acellular ECM from the delaminated bovine omasum. At 48 h, complete acellularity with no cellular debris was observed. No nuclear bodies were seen and the tissue was primarily composed of extracellular matrix. The propria-submucosa layer was completely acellular. The collagen fibers were thick and arranged in longitudinal and transverse manner as compared to the native tissue [79]. Sodium deoxycholate is very effective for removal of cellular components from tissue. Compared to other detergents, SD yields complete removal of nuclear remnants and cytoplasmic proteins, such as vimentin [80]. The extraction protocol for decellularization of delaminated bovine reticulum by 0.5% sodium deoxycholate treatment for 48 h has been documented. The concentration of native bovine omasum DNA ($P<0.01$) was 82.40 ± 1.41 ng/µl. Treatment with 0.5% SD showed lowest values 4.30 ± 0.14 ng/µl of DNA content in bovine omasum. Significant decrease ($P<0.01$) in DNA contents showed the effectiveness of treatment for decellularization. Significant decrease ($P<0.05$) in DNA contents was also observed while preparing acellular cholecyst matrices from buffalo origin [81]. Treatment with sodium deoxycholate showed effective removal of cells. Quantification of residual DNA in animal- derived biological scaffold materials is one of technical specifications for evaluating decellularization process and immunogenicity risk [77].

7.1. Preclinical evaluation

An acellular omasal derived extracellular matrix (b-AOM) of bubaline origin was prepared using anionic biological detergent. Healing potential of this matrix was compared with commercially available collagen sheet (b-CS) and open wound in full-thickness skin wounds in rats. Thirty-six clinically healthy adult Sprague Dawley rats of either sex was randomly divided into three equal groups. Under general anesthesia, a full-thickness skin wound (20 × 20 mm^2) was created on the dorsum of each rat. The defect in group I was kept as open wound and was taken as control. In group II, the defect was repaired with commercially available collagen sheet (b-CS). In group III, the defect was repaired with acellular omasal derived extracellular matrix (b-AOM). The wounds of groups II and III were dressed with standard dressing material [82].

Planimetry, wound contracture, immunological and histological observations were carried out to evaluate healing process. The colour digital photography of the wounds showed that on day 14, the graft was firmly adhered with underlying pinkish granulation tissue in group III. On day 28, the wound healed up completely with no scar in group III. There was crust over the wound area in groups I and II animals up to day 14. However, in groups I and II wound completely healed up on day 28 with severe contraction leaving a large scar over the healed area. A significant decrease ($P<0.05$) in wound area was recorded in all the groups on day 45, except in group III where complete healing was observed on day 28. Significant increase ($P<0.05$) in percent contraction was observed in all groups during observation period. On day 28, maximum wound contraction (100%) was recorded in group III animals [82].

The humoral response in rats elicited by application of different types of materials was determined by indirect ELISA. In all the treatment groups there were relative rise in antibody titre from day 0, 21 to day 28-post implantation. The B-cell responses were highest in the group II (ethanol dried bovine omasum) on day 21 as evidenced by higher absorbance values when compared with other groups. However, in group III, B-cell responses were highest on day 28. The test sera of group III animals show humoral immune response more or like as collagen group II animals. The cell mediated immune response in all groups was assessed by MTT colorimetric assay. At day 45, the SI value against the collagen sheet and native bovine omasum antigen the groups II and III showed significant ($P<0.05$) amount of stimulation control group. As compared to SI values of ConA all the groups exhibited considerable rise in SI values. The T-cell response was lowest in group III (implanted with ethanol dried acellular bovine omasum matrix) as evidenced by lower SI values [82].

In histopathological observations biopsy samples were examined with routine H & E staining and special staining for collagen with Masson's trichrome stain. In terms of surface epithelium presence and thickness, the group III showed early epithelialization with an early proliferation of fibroblast and angiogenesis to fill the defects. This group showed an early resemblance to the normal skin without any contraction than other groups. The histopathological scores were minimum in groups III followed by groups II and I. These findings indicate that b-AOM have potential for biomedical applications for full thickness skin wound repair in rats [82].

7.2. Clinical evaluation

The developed bubaline omasal derived extracellular matrix was clinically evaluated as a biological dressing material in dogs [83]. The study was conducted on five client-owned dogs (three males and two females), weighing from 14 kg to 24 kg and between 2 to 8 years of age having burn wounds either on the dorsum and on the limbs. In each case, before implantation of the MDM, owners were informed, and a written consent was obtained. It was decided to treat the wounds with bubaline omasal derived extracellular matrix prepared as per technique described by Singh *et al* [79]. Under sedation the wounds were debrided and the bubaline omasal derived extracellular matrix was applied on the wounds and secured with interrupted sutures of nylon. The matrix was covered with non-adherent surgical bandages. The bandages were changed on every alternate day or as per condition of surgical wounds. All the animals have excellent wound healing between 15 to 34 days. Based on this study, it can be concluded that this developed bubaline omasal derived extracellular matrix can be used as a biological dressing material in dogs. The developed matrix may be tried in other animal species also [83].

8. Preparation of acellular matrix from gall bladder

The cholecyst (gall bladder) is part of the digestive tract, and it lies within the abdominal cavity. The gall bladder is a sac like organ. The buffalo gallbladder (BG) was collected from local abattoir. Immediately after collection the gall bladder was kept in normal saline solution containing 0.02% EDTA and antibiotic (amikacin 1 mg/ml). The neck and fundus of the gallbladder was trimmed, followed by a longitudinal incision to obtain a flat sheet of tissue. The inner mucosal layer was peeled off and outer serosal layer was removed by mechanical delamination with a blunt edge. The tissue was cut into 2 × 2 cm^2 size pieces and treated with 70% ethanol for 2 h and later with distilled water for 24 h.

The BG tissue was treated with 0.5% and 1% sodium dodecyl sulphate (SDS) (ionic biological detergent) and 0.5% and 1% triton x-100 (non-ionic biological detergent) for 12 h, 24 h, 48 h and 72 h. The tissue was thoroughly washed in phosphate buffer saline solution after the completion of protocols. Acellularity of the b-CEM was evaluated by microscopic and scanning electron microscopic observations. The DNA concentration of native buffalo gallbladder and b-CEM extracellular matrices were evaluated [81,84].

Microscopically, native BG showed cellularity, mucosal and serosal layer, fatty tissue, and some liver portion. The delaminated sub mucosal layer showed cellularity, loose muscular layer, and collagen fibers. Masson's trichrome staining for native BG showing dense compact arrangement of collagen fibers (Figure 10a,b). The delaminated gallbladder treated with 0.5% SDS for 12 h under constant agitation showed loss of cellularity. The sub mucosal layer was completely acellular. The collagen fibers were compact with moderate porosity than the native tissue. Mild debris was observed between the spaces of thick collagen fibers. The collagen fibers became thin, compact and porosity was unchanged at 24 h. At 48 h b-CEM showed thin, heavily loose arranged collagen fibers with very high porosity. No debris was evident (Figure 11a,b). After 72 h, 0.5% SDS treated b-CEM was completely damaged with high porosity. No cellular debris was found. Delaminated 1% SDS treated

CEM at 12 h showed thick collagen fibers with moderately loose arranged collagen bundles with moderate cellular debris. At 24 h collagen fibers were loosely arranged with moderate porosity. In 1% SDS, at 48 h collagen fibers were heavily loose arranged with no cellular debris. At 72 h heavily loose, thin damaged collagen fibers with high porosity were observed [81,84].

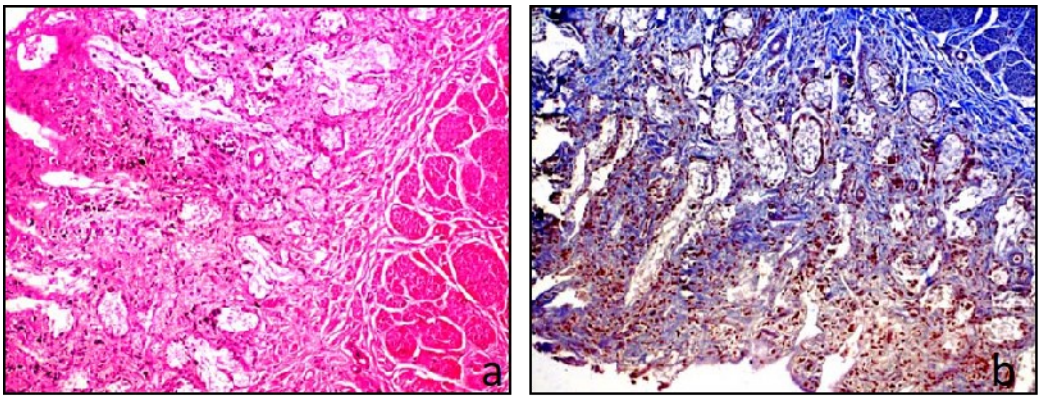

Figure 10. (a) Microscopic appearance of native bovine gall bladder showing cellularity, fatty tissue, mucosal and serosal layers, a-H&E stain, x-100, **(b)** Masson's trichrome stain, x-100 [28].

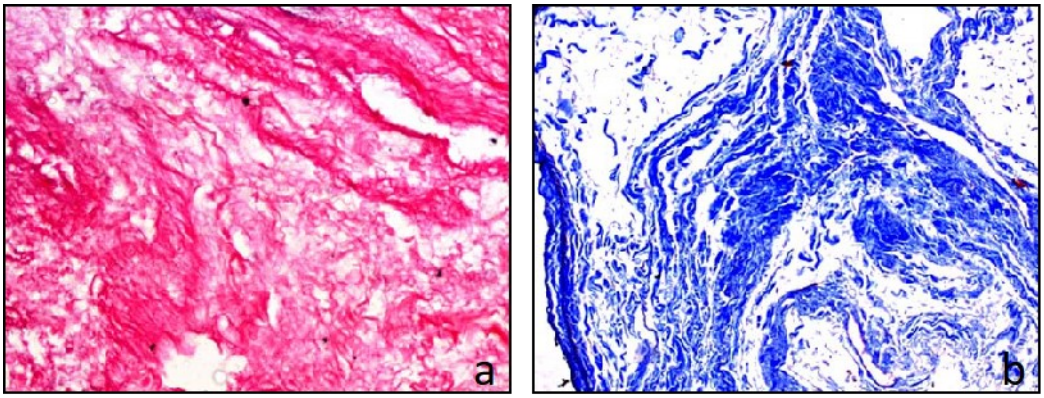

Figure 11. Microscopic appearance of acellular bovine gall bladder, **(a)** H&E stain, x-100, **(b)** Masson's trichrome stain, x-100 [28].

The delaminated CEM subjected to 0.5% triton X-100 for 12 h showed thick damaged collagen fibers with moderate cellular debris and porosity. At 24 h the collagen fibers were thin and mildly loose with high porosity. The collagen fibers were thin, loosely arranged, and damaged with increased porosity at 48 h and 72 h. In 1% triton X-100 treatment at 12 h, 24 h, 48 h and 72 h collagen fibers were loosely arranged and damaged from beginning with no cellular debris.

All the specimens treated for 12 h, 24 h, 48 h and 72 h were adequately decellularized and the best scaffold was prepared by treatment with 0.5% SDS. On Masson's trichrome staining thick, transversely, and longitudinally arranged collagen fibers were observed. The specimens treated with 1% SDS and triton X-100 (0.5% and 1%) were similar and showed very loose arrangement with damage of collagen fibers [81,84]. Scanning electron microscopic observations of native buffalo gall bladder showed dense and closely packed

collagen fibers. Collagen fibers were loosely arranged in decellularized b-CEM as compared to native buffalo gall bladder tissue. The collagen bundles were seen to be formed of many thick collagen fibrils. In the native gallbladder, abundant cell components and nucleic acids were apparent (Figure 12a–c) [81]. Scanning electron microscopic observations revealed loss of cellularity. However, after the decellularization, cells and nucleic acids were hardly observed in ECM. Collagen fibers were loosely arranged in decellularized b-CEM as compared to native buffalo gall bladder tissue. The collagen bundles were seen to be formed of many thick collagen fibrils (Figure 13a–c). The concentration of native buffalo gallbladder DNA ($P<0.05$) was 39.1 ηg/µl. After decellularization by different protocols, the DNA contents decreased and its ranges from 8.87 ± 0.33 ng/µl to 10.67 ± 0.73 ng/µl. Treatment with 0.5% SDS showed lowest values (8.87 ± 0.33 ng/µl) of DNA contents [81,84]. Protocols for decellularization of the buffalo gall bladder with ionic biological detergent (0.5% and 1% SDS) and non-ionic biological detergent (0.5% and 1% triton x-100) for different time intervals. Treatment with 0.5% sodium dodecyl sulphate for 48 h resulted in complete decellularization of buffalo gall bladder [81,84].

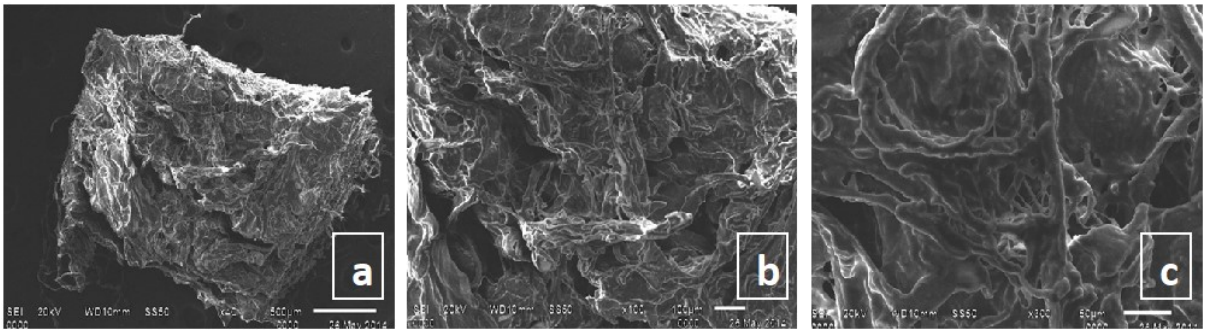

Figure 12. Scanning electron microscopic appearance of nativebovine gall bladder, **(a)** 40x, **(b)** 100x, **(c)** 150x [28].

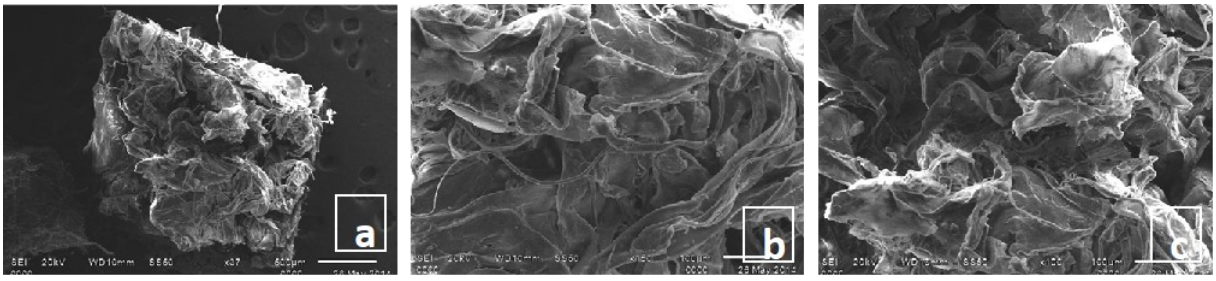

Figure 13. Scanning electron microscopic appearance of decellularized bovine gall bladder, **(a)** 40x, **(b)** 100x, **(c)** 150x [28].

8.1. Preclinical evaluation

Healing potential of b-CEM was compared with commercially available collagen sheet (b-CS) and open wound in full thickness skin wounds in rats. Thirty-six clinically healthy adult Sprague Dawley rats of either sex was randomly divided into three equal groups. Animals were anesthetized using xylazine (5 mg/kg body weight) and ketamine (50 mg/kg body weight) combination [85]. A full thickness skin wound (20 × 20 mm^2) was created on the dorsum of each rat. The defect in group I was kept as open wound and was taken as control. In group II, the defect was repaired with commercially available collagen sheet (b-CS) (Skin Temp-II, Absorbable Bovine Collagen, Polyethylene Non-Woven Mesh, Human BioSciences India Limited, 142/143P Vasna Chacharwadi, Ahmedabad 382213, India). In group III, the defect was repaired with cholecyst derived extracellular matrix of bubaline origin (b-CEM). Wound contracture, planimetry, hematological, immunological, and histological observations were carried out to evaluate healing process.

Wound area of all groups was measured by tracing its contour using transparent sheets with graph paper on postoperative days 0, 3, 7, 14, 21, and 28. Colour photographs were taken on post implantation days 7, 14, 21, and 28 with the help of digital camera at a fixed distance. Analysis of shape, irregularity, and colour of the lesion were determined. Blood smears for total leukocyte count (TLC) and differential leukocyte count (DLC) were prepared on postoperative days 0, 3, 7, 14, 21, and 28. The counts were expressed in percent. For the ELISA procedure serum was collected on days 0 and 28 from each rat. Harvested sera were assessed for the extent of antibodies titer generated toward the implant. The cell mediated immune response was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The stimulation of rat lymphocytes with phytohemagglutinin (PHA) was considered as positive control, whereas un stimulated culture cells were taken as negative control. The biopsy specimen from the implantation site was collected on 3, 7, 14, 21, and 28 post implantation days for histopathological evaluation [81,84].

The wound area decreased significantly ($P<0.05$) at various time intervals in different groups. However, the rate of decrease in wound area was faster in group III as compared to other groups, but there was no significant difference ($P>0.05$) between groups III and I in later stages (day 28). A significant increase ($P<0.05$) in percent contraction was observed in all groups during the observation period. On day 28, maximum wound contraction (98.92%) was recorded in group III animals where b-CEM was used. Although the original wound area created was 20 × 20 mm^2, the wounds were expanded to various extents in groups I and II because in group I no matrix was used and the wound healed as open wound and in group II commercially available collagen sheet was applied, and as it is very fragile in nature suturing was not possible. In group III prepared acellular cholecyst was sutured over the wound area to prevent drying and desiccation of the inner matrix. The wound area decreased gradually as the healing progressed. Maximum percent contraction was recorded in group I as no biomaterials were applied and wound healed as open wound. The wound in animals of this group healed completely by 28 days leaving a large scar indicating the existence of severe contraction. In group III the healing occurred with minimal contraction as the matrix was

sutured with wound edges in this group and collagen sheet was simply applied over the wound area [81,84].

During the entire period of the study none of the wounds showed any visible suppurative inflammation. Furthermore, none of the animals became sick or died. Graft-assisted healing is an important strategy for treating full thickness skin wounds. The cholecyst derived scaffold was rich in natural biomolecules like elastin and glycosaminoglycans and when used as a xenograft, it promoted healing with excess cell proliferation at early phases and acceptable collagen deposition in the later remodeling phases [86]. A significant (P<0.05) increase in neutrophils percentage after operation was observed up to day 3 postoperatively in all the groups. Thereafter, they started decreasing and reached within normal limits on day 21. Contrary to increased neutrophil count, a significant (P<0.05) decrease in lymphocyte count was observed up to day 7 in animals of all the groups postoperatively. The values fluctuated within the normal physiological range (65%–85%) in various groups at different time intervals.

The free protein contents of native bubaline gallbladder, decellularized bubaline gallbladder (b-CEM), and commercially available collagen sheet (b-CS) were estimated as per the methods of Lowry *et al* [87] using bovine serum albumin (BSA) as a standard. The values of protein contents of native bubaline gallbladder, b-CEM, and b-CS are 3.76 mg/mL, 0.054 mg/mL, and 0.06 mg/mL, respectively. The levels of antibodies present in serum prior to implantation were taken as basal values. In control group no rise in antibody titer was observed as the wound healed open without any matrix. In the treatment groups II and III there was relative rise in antibody titer from day 0 to day 28 after implantation. The B-cell response was significantly higher (P<0.05) in the groups II and III on day 28 as evidenced by higher absorbance values. As compared to the SI values of Con A and PHA all the groups exhibited significant (P<0.05) rise in SI values. The T-cell responses were lowest in the group III (implanted with acellular b-CEM) as evidenced by lower SI values. The immunological response to biomaterial depends on the nature of processing and presence of potentially foreign antigen to various lymphocyte populations. The biomaterial could stimulate an antibody response, a cell mediated sensitization, or minimal to no response. The immune response to xenogeneic transplantation included both natural and induced humoral components [88]. In the present study the animals of groups II and III showed slightly higher immune response on day 28 as compared to day 0. The least immune response was in animals of group I, where no graft was used. Slight immune response in groups II and III might be due to some immunogenic nature of decellularized ECM. The collagens are weakly immunogenic as compared to other proteins. However, complete elimination of alloantigen's is considered difficult to perform and verify [89]. The acellular grafts were less immunogenic having better tolerance by allogenic hosts and equally effective as isograft [90].

Full thickness skin wound healing occurs by granulation tissue formation, contraction, and epithelialization [91]. The epithelialization occurs by migration of undamaged epidermal cells from the wound margins across the granulation bed [92]. On day 7, after implantation, moderate to severe inflammation was present in all the groups and it was minimum in groups II and III. The early control of inflammation might be due to application of collagen matrix

as in case of groups II and III it might facilitate the progress to the next phase of wound healing. The proliferation of fibroblasts was more in group III. Similar findings were observed by Perme *et al* [93]. There was an increase in the extent of collagen deposition by 7 days (about 20%) and 14 days (about 40%) [86]. On day 14, the least scores were observed in groups III (21) followed by group II (26) which may be due to early acceptance of matrix. The sloughing of the upper layer of matrix was observed in all the groups except control group where wound remained open. It may be either due to the desiccation of the graft in high environmental temperature or due to an impaired formation of new blood vessels [94]. The myofibroblasts proliferate till the end of proliferation phase (14–15 days). Meanwhile, the underlying granulation tissue increased in mass that pushed up the graft upwards. The animals of groups II and III showed well-formed collagen and neovascularization with superficial epithelialization. The epithelialization and neovascularization were faster in group III as compared to other groups. On day 21, the least score was observed in group III (14) followed by group II (22). The epithelialization was more like the normal skin in the wounds of these groups. In the remodeling phase (around 21–30 days), these myofibroblasts undergo apoptosis [95]. On day 28, after implantation, the control group healed completely leaving abundant scar tissue but no complete healing was observed for other groups; also, in group II contraction and scar formation were found. In group III collagen fiber arrangement was almost like normal skin. Marked fibroblastic response associated with an abundant new collagenous fibrous tissue was observed in these groups. Histologically, improved epithelization, neovascularization, fibroplasia, and best arranged collagen fibers were observed in b-CEM (III) as early as on post implantation day 21. These findings indicate that b-CEM have potential for biomedical applications for full thickness skin wound repair in rats [81,84].

9. Preparation of acellular buffalo pericardium matrix

Fresh buffalo pericardium was procured from the local abattoir. Immediately after the collection the biomaterial was preserved in cold physiological saline solution. The tissue was gently rinsed with fresh saline to remove the adhered blood. Excess fatty layer was carefully removed from the pericardial surface. The biomaterials were cut into 2 × 2 cm^2 size pieces and were placed in a 20 ml of distilled water and shaken for 12 h to lyse the cells and to release the intracellular contents. It was followed by suspension of tissues in 4% sodium deoxycholate (SD) for 2 h to 4 h, then it was treated with 2000 Kunitz unit's DNase-I suspended in 1M sodium chloride solution and continuously stirred for 4 h. The process was repeated twice. Finally, the tissue was thoroughly washed in phosphate buffer saline (PBS) solution. The prepared acellular biomaterials were stored in PBS solution containing 1% amikacin at 4 °C. The tissue was also preserved in 10% formal saline solution for histological examination to confirm a cellularity [93,96].

The tissue matrices were examined grossly at desired time intervals. The gross observations of tissues showed that acellular pericardium was slightly softer and spongy as compared to native pericardium. 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 5µ thick paraffin sections. The sections were stained with hematoxylin and eosin and evaluated microscopically. Microscopic observation of acellular pericardium of buffalo revealed that the protocol for making them acellular was effective. It resulted in complete removal of cells. No nuclear bodies were seen. The tissue was primarily composed of ECM (Figure 14a,b) [93,96]. The primary constituent of pericardium is collagen which is essentially polymers of amino acids. The collagen molecule consists of 3 chains of poly amino acids or polypeptides arranged in a trihelices configurations ending in a non-helical carboxyl and amino terminals one at each end. These non-helical ends are believed to contribute to most of the antigenic properties of collagen. In natural state the collagen trihelical configurations are held in place by direct chemical bonds, hydrogen bonds and water-bridged cross-links.

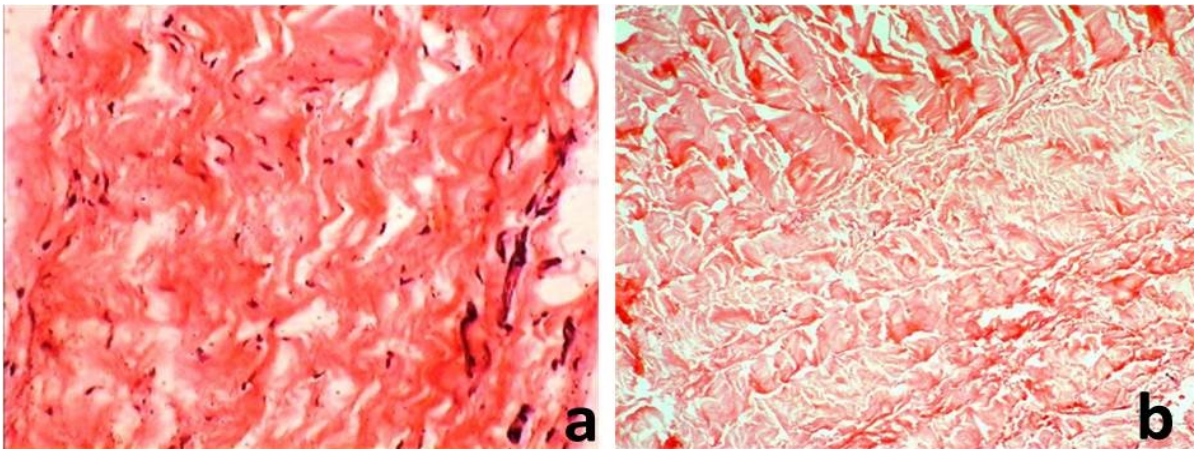

Figure 14. (a) Native buffalo pericardium; **(b)**acellular buffalo pericardium (H&E, stain, x100).

9.1. In vitro biocompatibility evaluation of pericardium

Collagenase enzymatic degradation. The degradation of the pericardium samples was studied by exposing the materials to collagenase [97]. Collagenase Type-I from *Clostridium histolyticum* (Sigma Aldrich Co., St Louis, MO, USA, C0130), 20 U/ml in phosphate buffered saline was used for enzymatic degradation at 12 h, 48 h, and 72 h. The tissues were blotted and the mass was recorded. Weight loss of biomaterial was then calculated that of the original tissue. The native pericardium showed higher percentage of weight loss ($P<0.01$) as compared to acellular and control samples. The study of enzymatic degradation can be a good model for evaluation of the resorption rate in tissue.

Free amino group contents determination. Native and acellular pericardium (1 gm each) was weighed, minced with scissors, and washed thrice with cold normal saline and was homogenized in 3 ml 10% sodium dodecyl sulphate (SDS) with pastel mortar. The homogenate was centrifuged at 3000 rpm for 10 min. and supernatant was separated. Ninhydrin assay was used to determine the free amino group contents of each test sample after enzymatic degradation, as per the procedure of Sung *et al* [98]. The free amino group contents determination for native and acellular pericardium showed significant ($P<0.01$)

reduction in amino group contents at 12 h, 48 h, and 72 h interval in acellular pericardium as compared to native and control [93,97]. It is also known that the reduction of free amino groups (determined by ninhydrin assay in the present study) in biological tissue diminishes its antigenicity [99].

Moisture content analysis. The moisture content was analyzed as per the method Sung *et al* [98]. In the study the moisture content of acellular tissue was significantly higher ($P<0.005$) than the native fresh tissue. The acellular tissues were found to be soft in consistency which may be attributed to their more absorption of moisture [93,96]. The moisture content of acellular tissue was recorded to be significantly higher than the native fresh tissue [98].

Molecular weight analysis. The tissues samples were mixed with an equal volume of 5x non-reducing sample buffer and using 10% poly-acrylamide gel under non-reducing condition as per method described by Laemmli [100]. SDS-PAGE of native pericardium showed 150 kDa, 120 kDa, 50 kDa and 20 kDa protein bands. Acellular pericardium showed only one protein bands between 60 kDa to 50 kDa. Native pericardium expressed a 72 kDa (MMP-2) band; whereas, in acellular pericardium two bands (10 kDa and 92 kDa) of MMPs were observed of which, 92 kDa band was very faint [93,96]. In native pericardium group, the 92 kDa band was the dullest among the three groups (Figure 15). This indicated that the level of MMP-9 corresponds to the degree of collagen degradation [101].

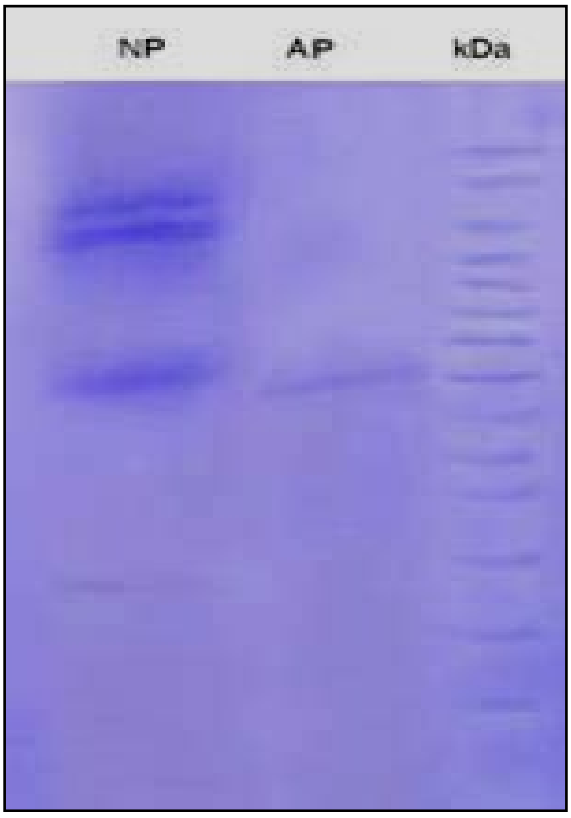

Figure 15. Native and acellular pericardium in SDS-PAGE, Lane 1-Native pericardium, Lane 2-Acellular pericardium, Lane 3-Marker [24].

In-vitro cell cytotoxicity. *In-vitro* cell cytotoxicity of biomaterials was done as per the method described by Goswami *et al* [102]. The cytotoxicity of native pericardium and acellular pericardium was tested in peripheral blood mononuclear cell (PBMC) culture.The SI of Con A (1.276 ± 0.0791) was used as control to evaluate the imuuno-suppression of biomaterials with and without Con A. The stimulation index revealed that the biomaterials when used with Con A showed moderate suppression of blastogenic effect of Con A, while suppression was greater when biomaterial was used alone without Con A. As there is ethical hindrance for indiscriminate use of animal models to test the cytotoxic effect biomaterials, the alternative approach is *in-vitro* cytotoxicity examination before *in-vivo* implantation study. The stimulation index (SI) revealed that the biomaterials when used with ConA showed moderate suppression of blastogenic effect of ConA, while suppression was greater when biomaterial was used alone without ConA [93,96].

In the present study the peripheral blood mononuclear cell (PBMC) was used to detect the immunosuppressive effect of biomaterials. The result indicated that the biomaterials with and without Con A showed immunosuppression as seen by the stimulation index when it was compared with the Con A which was used as control. Decellularization process may attenuate severe xenogenic immune response [103], but the removal of cellular components may not be sufficient to eliminate inflammation, and fixation techniques may still be necessary to prevent degradation [104]. It was hypothesized that cell extraction process decreased the antigenic load within the tissue due to the elimination of cellular antigens.

9.2. Preclinical evaluation

Fresh bovine pericardium procured from the local abattoir was cut into 2 × 2 cm^2 size pieces and crosslinked with 1% solution of glutaraldehyde (GA), formaldehyde (FA), polyethylene glycol (PEG), hexamethylene di-isocyanate (HMDI) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). Other pieces were made acellular and then crosslinked with above chemical agents. *In vivo* biocompatibility of the biomaterials was determined by subcutaneous implantation in rabbits. The implants were retrieved back on day 14, 30 and 90 post-implantation and subjected to macroscopic and microscopic evaluation. The host inflammatory response, neovascular tissue formation (fibroblasts, fine capillaries), deposition of neo collagen and penetration of host inflammatory responses in the grafted matrix were evaluated.

For *in-vivo* study the cross-linked biomaterials were subcutaneously implanted in the back muscle of rabbits. Macroscopically all the retrieved biomaterials showed thick fibrous connective tissue covering at days 14, 30 and 90. The implanted biomaterials showed decrease in mass at day 14 and 30 as compared to its original mass before implantation. The decrease in mass of graft was more at 90 days post implantation which was found deeply seated within the host fibrous connective tissue [93,105].

The native and acellular pericardium treated with NSS, FA, and HMDC were resorbed. Whereas GA and EDC treated grafts were partially resorbed. There was complete resorption of native diaphragm treated with NSS, FA, PEG and HMDC, whereas, GA and EDC treated

grafts were present deep beneath the fibrous connective tissue, however they had significantly reduced in size due to resorption.

The biomaterials were retrieved at 14-, 30- and 90-days intervals for histological evaluation. Microscopic study revealed that at day 14 all the treated biomaterials showed severe inflammatory reaction characterized by infiltration of neutrophils, macrophage, and fibroblast, which decreased by day 30. The NSS treated biomaterials were found to incite more inflammatory reaction as compared to treated biomaterials. By day 30 there was persistence of inflammatory reaction in NSS and HMDC treated biomaterials whereas other cross-linked biomaterials showed decrease in inflammatory reaction. At 90 days all the retrieved graft showed host tissue incorporation with reorganization of collagen. On evaluation of these biomaterials (GA and EDC treated) in experimental rabbits did not show any abnormal reaction including the graft rejection. Acellular cross-linked biomaterials were found to be more biocompatible compared to its native counterpart. *In-vivo* study revealed all the biomaterials showed increase host response at day 14 which decreased at day 30 and 90. NSS and HMDC treated biomaterials showed more host inflammatory reaction as compared to GA, FA, EDC treated biomaterials. The acellular biomaterials treated with different cross-linking agents were less immunogenic compared to its native counterpart. The biomaterials retrieved on day 90 showed reorganization of graft with host tissue. Microscopic observation of acellular pericardium revealed that the protocol for acellular cell production was effective [93,105].

10. Summary and conclusions

Most of the collagen scaffolds used in tissue engineering belongs to porcine or bovine origin. Bovines are prone to different zoonotic diseases like bovine spongiform encephalopathy (BSE or Mad Cow disease), transmissible spongiform encephalopathy (TSE) and foot and mouth disease (FMD). The use of collagen from this species may transmit the dreadful diseases to the recipient. Therefore, collagen of bovine origin has limited use. The collagen scaffolds developed from porcine origin has little clinical acceptance in our country on cultural and religious grounds. More over the slaughtering of cattle is illegal/ban in most of the part of India. So, we must look the other sources of the collagen. The collagen scaffolds developed from bubaline (buffalo origin) allow greater clinical acceptance on cultural and religious grounds in addition to presenting a low risk with respect to viral pathogens and prions. Keeping in view of certain disadvantages, source of bovine (cattle) and porcine (pig) collagen may be replaced by bubaline (buffalo) collagen. The protocols for decellularization of collagen scaffolds from buffalo aorta, diaphragm, rumen, reticulum, omasum, skin, gall bladder and pericardium have been optimized. No methods of decellularization resulted in disruption of the architecture and loss of surface structure and composition. The developed acellular collagen matrices were preclinically tested in laboratory animals for their biocompatibility. Suitably found matrices were treated clinically in different species of animals for reconstructive surgery. The acellular ECM scaffolds can create a favorable regenerative microenvironment, promote tissue-specific remodeling, and act as an inductive

template for the repair and functional reconstruction. The construction of three-dimensional extracellular matrix scaffolds, and their tissue engineering applications, with a focus on translation of these novel tissue engineered products to the human applications.

11. Conclusions and future prospectives

Most of the collagen scaffolds used in tissue engineering belongs to porcine or bovine origin. Bovines are prone to different zoonotic diseases like bovine spongiform encephalopathy (BSE or Mad Cow disease), transmissible spongiform encephalopathy (TSE) and foot and mouth disease (FMD). The use of collagen from this species may transmit the dreadful diseases to the recipient. Therefore, collagen of bovine origin has limited use. The collagen scaffolds developed from porcine origin has little clinical acceptance in our country on cultural and religious grounds. More over the slaughtering of cattle is illegal/ban in most of the part of India. So, we must look the other sources of the collagen. The collagen scaffolds developed from bubaline (buffalo origin) allow greater clinical acceptance on cultural and religious grounds in addition to presenting a low risk with respect to viral pathogens and prions. Keeping in view of certain disadvantages, source of bovine (cattle) and porcine (pig) collagen may be replaced by bubaline (buffalo) collagen. No methods of decellularization resulted in disruption of the architecture and loss of surface structure and composition. The acellular ECM scaffolds can create a favorable regenerative microenvironment, promote tissue-specific remodeling, and act as an inductive template for the repair and functional reconstruction. The construction of three-dimensional extracellular matrix scaffolds, and their tissue engineering applications, with a focus on translation of these novel tissue engineered products to the human applications.

Conflicts of Interests

There is no conflict of interest among authors.

Authors' Contribution

All authors contributed equally in writing this article.

References

[1] Nelson CM, Bissell MJ. Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. *Annu Rev Cell Dev Biol* 2006, 22:287-309.

[2] Taylor KR, Gallo RL. Glycosaminoglycans and their proteoglycans: host associated molecular patterns for initiation and modulation of inflammation. *FASEB J* 2006, 20(1):9-22.

[3] Badylak SF. The extracellular matrix as a biologic scaffold material. *Biomaterials* 2007, 28:3587-93.

[4] Barkan D, Green JE, Chambers AF. Extracellular matrix: a gatekeeper in the transition from dormancy to metastatic growth. *Eur J Cancer* 2010, 46 (7):1181-8.

[5] Vorotnikova E, McIntosh D, Dewilde A, Zhang J, Reing JE, *et al*. Extracellular matrix-derived products modulate endothelial and progenitor cell migration and proliferation *In vitro* and stimulate regenerative healing *in vivo*. *Matrix Biol* 2010, 29(8):690-700.

[6] Badylak SF. Xenogeneic extracellular matrix as a scaffold for tissue reconstruction. *Transpl Immunol* 2004, 12:367-77.

[7] Badylak SF, Gilbert TW. Immune response to biologic scaffold materials. *Semin Immunol* 2008, 20:109-16.

[8] Singelyn JM, Christman KL. Injectable materials for the treatment of myocardial infarction and heart failure: the promise of decellularized matrices. *J Cardiovas Translat Res* 2010, 3: 478-86.

[9] Badylak SF. Decellularized allogeneic and xenogeneic tissue as a bioscaffold for regenerative medicine: factors that influence the host response. *Annals Biomed Engineering* 2014, 42:1517-27.

[10] Irvine SM, Cayzer J, Todd EM, Lun S, Floden EW, Negron L. Quantification of *In vitro* and in vivo angiogenesis stimulated by ovine forestomach matrix biomaterial. *Biomaterial* 2011, 32:6351-61.

[11] Stump A, Holton LH, Connor J, Harper JR, Slezak S, Silverman RP. The use of acellular dermal matrix to prevent capsule formation around implants in a primate model. *Plastic Reconst Surg* 2009, 124(1):82-91.

[12] Buinewicz B and Rosen B. Acellular Cadaveric Dermis (AlloDerm): A new alternative for abdominal hernia repair. *Annals Plastic Surg* 2004, 52(2):188-194.

[13] Sarikaya A, Record R, Wu CC, Tullius B, Badylak S, *et al*. Antimicrobial activity associated with extracellular matrices. *Tissue Eng* 2002, 8: 63-71.

[14] Simionescu DT, Lovekamp JJ, Vyavahare NR. Degeneration of bioprosthetic heart valve cusp and wall tissues is initiated during tissue preparation: an ultrastructural study. *J Heart Valve Dis* 2003, 12(2):226-34.

[15] Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: structure and function. *Acta Biomater* 2009, 5(1):1-13.

[16] Allen RA, Seltz LM, Jiang H, Kasick RT, Sellaro TL, *et al*. Adrenal extracellular matrix scaffolds support adrenocortical cell proliferation and function *In vitro*. *Tissue Eng Part A* 2010, 16(11):3363-74.

[17] Hubbell JA. Biomaterials in tissue engineering. *Biotechnology* 1995, 13(6):565-76.

[18] Hendow EK, Guhmann P, Wright B, Sofokleous P, Parmar N, *et al*. Biomaterials for hollow organ tissue engineering. *Fibrogenesis Tissue Repair* 2016, 9:3.

[19] Hodde J. Naturally occurring scaffolds for soft tissue repair and regeneration. *Tissue Eng.* 2000, 8:295-308.

[20] Chan BP, Leong KW. Scaffolding in tissue engineering: general approaches and tissue-specific considerations. *Eur Spine J* 2008, 17 Suppl 4:467-79.

[21] Gu X. Progress and perspectives of neural tissue engineering. *Front Med* 2015, 9(4):401-11.

[22] Sheng Y, Ding F, Gong L, Gu X. Extracellular matrix scaffolds for tissue engineering and regenerative medicine. *Curr Stem Cell Res Ther* 2017, 12:233-246.

[23] Singh J, Kumar N, Sharma AK, Maiti SK, Goswami TK, *et al*. Acellular biomaterials of porcine origin for the reconstruction of abdominal wall defects in rabbits. *Trends Biomat Artif Organs* 2008, 22:33-44.

[24] Perme H, Sharma AK, Kumar N, Singh H, Dewangan R, *et al*. *In-vitro* biocompatibility evaluation of crosslinked cellular and acellular bovine pericardium. *Trends Biomat Artif Organs* 2009, 23:65-75.

[25] Kumar V, Kumar N, Gangwar AK, Saxena AC. Using acellular aortic matrix to repair umbilical hernias of calves. *Australian Vet J* 2013, 91(6):251-253.

[26] Kumar V, Kumar N, Singh H, Mathew DD, Singh K, *et al*. An acellular aortic matrix of buffalo origin crosslinked with 1-ethyl-3-3-dimethylaminopropylcarbodiimide hydrochloride for the repair of inguinal hernia in horses. *Equine Vet Edu* 2013, 25(8):398-402.

[27] Kumar V, Kumar N, Gangwar AK, Singh H. Comparison of acellular small intestinal matrix (ASIM) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide crosslinked ASIM (ASIM-EDC) for repair of full-thickness skin wounds in rabbits. *Wound Med* 2014, 7(1):24-33.

[28] Shakya P, Sharma AK, Kumar N, Remya V, Mathew DD, *et al*. Bubaline cholecyst derived extracellular matrix for reconstruction of full thickness skin wounds in rats. *Scientifica* 2016.

[29] Singh P, Sharma AK, Kumar N, Tamil Mahan P, Sangeetha P, *et al*. Process development to prepare an acellular matrix from bovine omasum using biological detergents and enzymes. *Trends Biomat Artif Organs* 2017, 31(1):2-8.

[30] Singh AK, Kumar N, Shrivastava S, Singh KP, Dey S, *et al*. Evaluation of the efficacy of bubaline rumen derived extracellular matrix in umbilical hernioplasty in buffalo calves. *International J Fauna Biol Studies* 2018, 5(1):29-33.

[31] Kumar V, Vora SD. Abdominal wall repair with bubaline aortic matrix in a goat. *Indian J Small Ruminants* 2019, 25(2):255-256.

[32] Kumar N, Devarathnam J, Sharma AK, Singh H, Kumar V, *et al*. Optimization of protocols for decellularization of aortic matrix of buffalo origin, *Presented in XXIV annual congress of ISVS and International symposium on newer concepts in surgical techniques for farm and companion animal practice held at Puduchery* on 8-10 December 2010 pp 117.

[33] Devarathnam J, Sharma AK, Kumar N, Rai RB. Optimization of protocols for decellularization of buffalo aorta. *J Biomat Tissue Engineering* 2014, 4(10):778-785.

[34] Devarathnam J. Biocompatibility determination of acellular aortic matrix of buffalo origin. *MVSc thesis submitted to Deemed University Indian Veterinary Research Institute*, Izatnagar-243122, 2010, Uttarpradesh, India.

[35] Vora SD, Kumar V, Singh VK, Fefar DT, Gajera HP. Bubaline aortic matrix: Histologic, imaging, Fourier transform infrared spectroscopic characterization and application into

cattle abdominal hernia repair. Proceedings of the National Academy of Sciences, *India Section B: Biological Sciences* 2020, 90(1):161-170.

[36] Chang MC, Tanaka J. FTIR study for hydroxyapatite/collagen nanocomposite cross-linked by glutaraldehyde. *Biomaterials* 2002, 23:4811-4818.

[37] Roy S, Silacci P, Stergiopulos N. Biomechanical proprieties of decellularized porcine common carotid arteries. *Am J Physiol Heart Circ Physiol* 2005, 289:1567-76.

[38] Sharma AK, Devarathnam J, Kumar N, Gangwar AK, Kumar V, *et al*. *In-vivo* biocompatibility determination of acellular aortic matrix of buffalo origin *Presented in XXIV annual congress of ISVS and International symposium on "Newer concepts in surgical techniques for farm and companion animal practice"* held at Puduchery on 8-10 December 2010.

[39] Devarathnam J, Sharma AK, Kumar N, Shrivastava S, Sonal B, *et al*. *In vivo* biocompatibility determination of acellular aortic matrix of buffalo origin, *Prog Biomater* 2014, 3:115–122.

[40] Devarathnam J, Sharma AK, Rai RB, Maiti SK, Shrivastava SK, *et al*. *In vitro* biocompatibility determination of acellular aortic matrix of buffalo origin. *Trends Biomat Artific Organs* 2014, 23(3):92-98.

[41] Kumar V, Devarathnam J, Gangwar AK, Kumar N, Sharma AK, *et al*. Use of acellular aortic matrix for reconstruction of abdominal hernias in buffaloes. *Vet Rec* 2012, 170(15):392.

[42] Devarathnum J, Sharma AK, Gangwar AK, Kumar V, Singh H, *et al*. Acellular aortic grafts for the reconstruction of umbilical hernias in Holstein-Friesian calves. *Vet Pract* 2012,13(2):307-308.

[43] Kumar V, Kumar N, Devarathnam J, Pawde AM, Gangwar AK, *et al*. Successful tracheal replacement with acellular aortic graft in a cow. *J Vet Advances* 2012, 2(11):552-556.

[44] Kumar V, Mathew DD, Kumar N, Singh K, Ahmad RA. An acellular aortic matrix of buffalo origin crosslinked with 1-ethyl-3-3-dimethylaminopropylcarbodiimide hydrochloride for the repair of inguinal hernia in horses. *Equine Vet Edu* 2013, 398-402.

[45] Gangwar AK, Kumar V, Devarathnum J, Kumar N, Sharma AK, *et al*. Acellular aortic matrix for the repair of large ventral hernia in a buck. *Vet Pract* 2012, 13(2):293-294.

[46] Kumar V, Gangwar AK, Kumar N, Singh H. Use of the bubaline acellular diaphragm matrix for umbilical hernioplasty in pigs. *Veterinarski Arhiv* 2015, 85(1):49-58.

[47] Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularisation processes. *Biomaterials* 2011, 32:3233-43.

[48] Kumar V, Gangwar AK, Mathew DD, Ahmad RA, Saxena AC, *et al*. Acellular dermal matrix for surgical repair of ventral hernia in horses. *J Equine Vet Sci* 2013, 33(4):238-43.

[49] Kumar V, Gangwar AK, Kumar N. Evaluation of the murine dermal matrix as a biological mesh in dogs. *Proc Natl Acad Sci, India, Sect B Biol Sci* 2016, 86(4):953-60.

[50] Seddon AM, Curnow P, Booth PJ. Membrane proteins, lipids and detergents: not just a soap opera. *Biochem Biophys Acta* 2004, 1666:105-17.

[51] Woods T, Gratzer PF. Effectiveness of three extraction techniques in the development of a decellularized bone-anterior cruciate ligament-bone graft. *Biomaterials* 2005, 26:7339-49.

[52] Singh H, Kumar N, Sharma AK, Kumar A, Dewangan R, *et al*. Host tissue response to subcutaneously implanted native and acellular scaffold in a rabbit model. *Trends Biomat Artific Organs* 2011, 25:37-42.

[53] Londono R, Dziki JL, Haljasmaa E, Turner NJ, Leifer CA, Badylak SF. The effect of cell debris within biologic scaffolds upon the macrophage response. *J Biomed Mater Res A* 2017, 105:2109-18.

[54] Kaarthick DT, Sharma AK, Kumar N, Kumar V, Gangwar AK, *et al*. Accelerating full-thickness dermal wound healing using primary mouse embryonic fibroblasts seeded bubaline acellular diaphragm matrix. *Trend Biomat Artific Organs* 2017, 31(1):16-23.

[55] Remya V, Kumar N, Saxena S, Shrivastava S, Sharma AK, *et al*. Mesenchymal stem cell tailored bioengineered scaffolds derived from bubaline diaphragm and aortic matrices for reconstruction of abdominal wall defects. *J Tissue Engineering Reg Med* 2020, 14(12):1763-1778.

[56] Rathore HS, Kumar N, Singh K, Maiti SK, Shrivastava S, *et al*. Clinical application of acellular matrix derived from the bubaline diaphragm and caprine rumen for the repair of abdominal wall defects in animals. *Aceh J Ani Sci* 2019, 4(2):50-60.

[57] Rathore HS, Raghuvanshi PDS, Gautam D, Mohan D, Singh AK, *et al*. Use of the bubaline acellular diaphragm matrix (ADM) for repair of abdominal wall defects in four different species of animals. *Inter J Chem Studies* 2018, 6(4): 157-161.

[58] Remya V, Shakya P, Sivanarayanan TB, Dubey P, Pawde AM, *et al*. Umbilical hernioplasty using acellular bovine diaphragm matrix in a calf. *Ruminant Sci* 2013, 2(2):229-231.

[59] Remya V, Mathew DD, Shakya P, Ahmad RA, Mohsina A, *et al*. Perineal hernioplasty using acellular diaphragm matrix in a male spitz dog. *Paper presented in XXXVII Annual Congress of Indian Society for Veterinary Surgery and National Symposium on Need for specilaization and Super-specialization in Veterinary Surgery and Imaging techniques for Professional Efficiency development* held at Mannuthy, Thrissur, Kerala from 6 to 8 November 2013 page 246.

[60] Mathew DD, Kumar N, Ahmad RA, Remya V, Vijaykumar H, *et al*. Hysterocele in a Dachshund dog and its surgical correction with decellularised diaphragm matrix. *Paper presented in XXXVII Annual Congress of Indian Society for Veterinary Surgery and National Symposium on Need for specilaization and Super-specialization in Veterinary Surgery and Imaging techniques for Professional Efficiency development* held at Mannuthy, Thrissur, Kerala from 6 to 8 November 2013, page 244.

[61] Mathew DD, Singh K, Gopinathan A, Mohsina A, Bodh D, *et al*. Inguinal hernioplasty using acellular diaphragm matix in a horse. *Paper presented in XXXVII Annual Congress of Indian Society for Veterinary Surgery and National Symposium on Need for specilaization and Super-specialization in Veterinary Surgery and Imaging*

techniques for Professional Efficiency development held at Mannuthy, Thrissur, Kerala from 6 to 8 November 2013 page 62.

[62] Remya V, Kumar N, Singh K, Gopinathan A, Shakya P, *et al*. *Acellular Diaphragm Matrix-A promising and immuno-tolerable prosthetic for hernioplasty. Paper presented in XXXVIII Annual Congress of Indian Society for Veterinary Surgery and International Symposium on New Horizons of Camel Surgery and Large Ruminant Surgery* held at COVS, RUVAS, Bikaner (Rajasthan) from 15 to 16 October 2014, page 360.

[63] Shakya P, Mathew DD, Remya V, Mohsina A, Sharma AK, *et al*. Acellular diaphragm matrix for the repair of umbilical hernia in two cow calves. *Paper presented in XXXVII Annual Congress of Indian Society for Veterinary Surgery and National Symposium on Need for specilaization and Super-specialization in Veterinary Surgery and Imaging techniques for Professional Efficiency development* held at Mannuthy, Thrissur, Kerala from 6 to 8 November 2013, page 189.

[64] Singh AK. Development of 3-D bioengineered scaffolds from bubaline rumen and gallbladder for the regeneration of full thickness skin wounds in diabetic rats. *PhD thesis submitted to Deemed University, Indian Veterinary Research Institute*, Izatnagar 2017, Uttar Pradesh, India.

[65] Singh AK, Kumar N, Om Preethi B, Shrivastava S, Gopinathan A, *et al*. Bubaline rumen derived scaffold for the regeneration of full thickness skin wounds in diabetic rats. Presented in 41st Annual Congress of ISVS on New horizons in cancer research pertaining to effect on health, production and reproduction in animals held at Tirupati, Andhra Pradesh from 14-16 December, 2017, SAS 38, Page 266.

[66] Mathew DD, Kumar N, Sharma AK, Remya V, Negi M, *et al*. *Development of collagen-based biomaterials from ruminant gastric wall for tissue engineering. Presented in 3rd World Congress on Biotechnology held at Hyderabad International Convention Centre*, Hyderabad, during September 13-15, 2012, Page 198.

[67] Singh AK, Kumar N, Shrivatava S, Singh KP, Dey S, *et al*. Umbilical hernioplasty using bubaline rumen derived extracellular matrix in bovine calves. *Presented in 41st Annual Congress of ISVS on New horizons in cancer research pertaining to effect on health, production and reproduction in animals held at Tirupati*, Andhra Pradesh from 14-16 December, 2017, RUS 47, Page 223-224.

[68] Hafez ESE, Badreldin AL, Shafei MM. Skin structure of Egyptian buffaloes and cattle with particular reference to sweat glands. *J Agricultural Sci* 2009, 46:19-30.

[69] Purohit S, Kumar N, Sharma AK, Sharma AK. Development of acellular dermal matrix from skin of different species of animals using biological detergents and enzymes combinations. *JSM Burns Trauma* 2016, 1:1004.

[70] Livesey SA, Campo AA, Nag A, Nichols KB, Coleman C. Method for processing and preserving collagen-based tissues for transplantation. US Patent#5,336,616 *Life Cell Corporation* 1994, 23-24.

[71] Weber RA, Proctor WH, Warner MR, Verheyden CN. Autonomy and the sciatic functional index. *Microsurgery* 1993, 14:323-329.

[72] Kumar V, Kumar N, Gangwar AK, Sharma AK, Singh H, *et al*. Acellular dermal grafts for the reconstruction of umbilical/ventral hernias in buffalo calves. *Vet Pract* 2012, 13:90-92.

[73] Chung S, Hazen A, Levine JP, Baux G, Olivier WA, *et al*. Vascularized acellular dermal matrix island flaps for the repair of abdominal muscle defects. *Plastic Reconstr Surg* 2003, 111:225-232.

[74] Gangwar AK, Sharma AK, Kumar N, Maiti SK, *et al*. Acellular dermal graft for repair of abdominal wall defects in rabbits. *J South African Vet Assoc* 2006, 77:79-85

[75] Ward BR, Johnson KD, Charles B, May H. Tissue scaffolds derived from forestomach extracellular matrix. Patent No US 8, 758, 781 B2, Date of patent, Jun. 24, 2014.

[76] Lun S, Irvine SM, Johnson KD, Fisher NJ, Floden EW, *et al*. A functional extracellular matrix biomaterial derived from ovine forestomach. *Biomaterials* 2010, 31:4517-29.

[77] Xu L, Shao A, Zhao Y. Quantification of residues DNA in animals-derived biological scaffold materials. *J Biomed Engineering* 2012, 29(3):479-85.

[78] Hasan A, Kumar N, Gopinathan A, Singh K, Sharma AK, *et al*. Bovine reticulum derived extracellular matrix (b-REM) for reconstruction of full thickness skin wounds in rats. *Wound Med* 2016, 12:19-31.

[79] Singh P, Sharma AK, Kumar N, Tamil Mahan P, Sangeetha P, *et al*. Process development to prepare an acellular matrix from bovine omasum using biological detergents and enzymes. *Trends Biomat Artific Organs* 2017, 31(1):2-8.

[80] Woods T, Gratzer PF. Effectiveness of three extraction techniques in the development of a decellularized bone-anterior cruciate ligament-bone graft. *Biomaterials* 2005, 26:7339-7349.

[81] Shakya P, Sharma AK, Kumar N, Remya V, Mathew DD, *et al*. Bubaline cholecyst derived extracellular matrix for reconstruction of full thickness skin wounds in rats. *Scientifica* 2016:2638371.

[82] Shakya P, Sharma AK, Kumar N, Vellachi R, Mathew DD, *et al*. Bubaline Cholecyst Derived Extracellular Matrix for Reconstruction of Full Thickness Skin Wounds in Rats. *Scientifica (Cairo)* 2016:2638371.

[83] Singh P, Sharma AK, Kumar N, Maiti SK, Tamil Mahan P, Rashmi, Shah MA, Sharma D, Basha AM, Sahu S, Shivaraju S. Reconstruction of full thickness burn wounds in dogs by using bubaline omasal derived extracellular matrix. *Presented in 41st Annual Congress of ISVS on New horizons in cancer research pertaining to effect on health, production and reproduction in animals held at Tirupati*, Andhra Pradesh from 14-16 December, 2017, SAS 51, page 274.

[84] Shakya P. Evaluation of cholecyst derived-extracellular matrix for reconstruction of full thickness skin wounds in rats. *MVSc thesis submitted to Deemed University Indian Veterinary Research Institute*, Izatnagar, 2015, Uttarpradesh, India.

[85] Stringer SK, Seligmann BE. Effects of two injectable anesthetic agents on coagulation assays in the rat. *Lab Ani Sci* 1996, 46(4):430–433.

[86] Revi D, Vineetha VP, Muhamed J, Rajan A, Anilkumar TV. Porcine cholecyst-derived scaffold promotes fullthickness wound healing in rabbit. *J Biomat Tissue Engineering* 2013, 3:261-272.

[87] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951, 193:265–275.

[88] Ruszczak Z. Effect of collagen matrices on dermal wound healing. *Adv. Drug Deliv. Rev.* 2003, 55(12):1595–1611.

[89] Malone JM, Brendel K, Duhamel RC, Reinert RL. Detergent-extracted small-diameter vascular prostheses. *J Vascul Surg* 1984, 1(1):181-191.

[90] Gulati AK, Cole GP. Nerve graft immunogenicity as a factor determining axonal regeneration in the rat. *J Neurosurg* 1990, 72(1):14–122.

[91] Fossum TW, Hedlund CS, Johnson AL *et al.* Surgery of Integumentary System, In Manual of Small Animal Surgery, Mosby, Philadelphia 2007, USA pp159–175.

[92] Swaim SF, Henderson RA. Wound Management, In Small Animal Wound Management. *Lea and Febiger* 1990, Vol. 9, page 33.

[93] Perme H, Sharma AK, Kumar N, Singh H, Dewangan R, *et al*. *In-vitro* biocompatibility evaluation of crosslinked cellular and acellular bovine pericardium. *Trends Biomat Artific Organs* 2009, 23(2):65-75.

[94] Boyce ST. Cultured skin substitutes: A review. *Tissue Eng.* 1996, 2(4):255-266.

[95] Li B, Wang JHC. Fibroblasts and myofibroblasts in wound healing: force generation and measurement. *J Tissue Viability* 2011, 20(4):108-120.

[96] Perme H. *In-vitro* and *in-vivo* biocompatibility of crosslinked bovine pericardium and diaphragm. *MVSc thesis submitted to Deemed University Indian Veterinary Research Institute*, Izatnagar, 2006, Uttarpradesh, India.

[97] Connolly JM, Alferiev I, Jocelyn N, Gruel C, Eidelman N, *et al*. Triglycidylamine cross-linking of porcine aortic valve cusps or bovine pericardium results in improved biocompatibility, biomechanics and calcification resistance. *Am J Path* 2005, 166(1):1-13.

[98] Sung HW, Chang Y, Liang IL, Chang WH, Chen YC. Fixation of biological tissues with a naturally occurring cross-linking agent: Fixation rate and effects of pH, temperature, and initial fixative concentration. *J Biomed Mat Res* 2000, 52:77-85.

[99] Imamura E, Sawatani O, Koyanagi H, Noishiki Y, Miyata T. Epoxy compounds as a new crosslinking agent for porcine aortic leaflets: Subcutaneous implants studies in rats. *J Cardiac Surg* 1989, 4:50-57.

[100] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. *Nature* 1970, 680-685.

[101] Singh H, Kumar N, Sharma AK, Kataria M, Munjal A, *et al*. Activity of MMP-9 after repair of abdominal wall defects with acellular and cross-linked bovine pericardium in rabbit. *Internat Wound J* 2012, 11(1):5-13.

[102] Goswami TK, Kumar N, Gupta OP, Sharma AK, Pawde AM. *In-vitro* determination of biocompatibility of different grafts/implants used for reconstructive surgery of tendon. *Indian J Ani Sci* 2002, 72:957-959.

[103] Goldstein S, Black K, Clarke D, Orton EC, O'Brien MF. Inflammatory response to uncrosslinked xenogenic heart valve matrix. *World Symposium on Heart Valve Disease London* 1999:205.

[104] Courtman DW, Wilson GJ. Development of an acellular matrix vascular xenograft: Modification of the in-vivo immune response in rats. *Proceedings of the Soceity for Biomaterials 25th Annual Meetings* 1999:20.

[105] Perme H, Sharma AK, Kumar N, Singh H, Maiti SK, *et al*. *In-vivo* biocompatibility evaluation of cross-linked cellular and acellular bovine pericardium. *Indian J Ani Sci* 2009, 79:658-661.