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RESEARCH PAPER

Clinical application of acellular matrix derived from the bubaline diaphragm and caprine rumen for the repair of abdominal wall defects in animals

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ABSTRACT

The abdominal wall hernias resulting due to trauma or other clinical conditions are common in animals. Large hernias required the use of synthetic mesh, which is costly and may result in infection, fistula formation, and pain. Application of biomaterials in hernia repair causes a reduction in pain, reduced recovery time, and rate of recurrence. The study was undertaken to test the acellular bubaline diaphragm matrix (BDiaM) and acellular caprine rumen matrix (CRuM) for the repaired hernia in clinical cases. Fresh bubaline diaphragm and caprine rumen were decellularized using sodium deoxycholate (1% for CRuM and 2% for BDiaM) for 48h. Acellularity was ascertained histologically and by DNA quantification. Histologically, both the matrices showed complete acellularity and orderly arranged collagen fibers after 48 h. The DNA contents were significantly ($P<0.05$) reduced in both the matrices in comparison to the native matrices. The BDiaM and CRuM matrices were applied in eight and nine clinical cases of abdominal wall defects, respectively. Animals with BDiaM and CRuM matrices recovered uneventfully and remained sound at least up to 3 months. Hematological and immunological findings were unremarkable. BDiaM and CRuM matrices showed good results without complications.

Keywords: Biocompatibility, Bubaline diaphragm matrix, Caprine rumen matrix, DNA quantification, ELISA, SDS-PAGE

INTRODUCTION

The abdominal wall defects due to trauma in animals are quite common (Ladurner *et al.,* 2001). Primary closure can repair small abdominal wall defects while hernias with massive defects, including irreducible hernia, requires special surgical procedures by the use of graft (Iqbal *et al.,* 1994). The synthetic mesh may cause infection, fistula formation, and pain. The acellular xenogenic and allogenic materials were used to avoid such postoperative complications (Khan *et al.,* 2008). Use of biomaterials in hernia repair causes a reduction in pain, less recovery time, and reduced rate of recurrence (Amid, 1997).

Synthetic non-absorbable meshes provide good mechanical strength but may cause intestinal adhesions and obstruction, wound infection, fistula formation, and seroma/hematoma formation (Eid *et al.,* 2003). Biological materials provided a framework for fibroblast proliferation and minimized adhesion formation (Clarke *et al.,* 1996). Extracellular matrix derived from mammalian tissues allows early incorporation and remodeling by the host tissue (Butler, 2006). The biomaterials

in their native form are more immunogenic, and their immunogenicity is minimized by decellularization (Gilbert *et al.,* 2006). Low immunogenicity and acceptance of acellular grafts in experimental animals were observed by Gulati and Cole (1994). Collagen grafts were found biocompatible, biodegradable, and have a weak antigenic reaction (Lee *et al.,* 2001).

Decellularization removes all cellular and nuclear material from the extracellular matrix (ECM) (Gilbert *et al.,* 2006) and has good mechanical strength, blood vessel and host cell incorporation (Schmidt and Baier, 2000). Extracellular matrix provides host cell attachment sites and encourages cell migration and proliferation (Badylak, 2007; Laschke *et al.,* 2009). Extracellular matrix as a biological scaffolds are isolated from pericardium (Perme *et al.,* 2009), diaphragm (Kaarthick *et al.,* 2017), skeletal muscle (Borschel *et al.,* 2004), small intestinal submucosa (Kropp *et al.,* 1995; Kumar *et al.,* 2013a), urinary bladder (Dewangan *et al.,* 2012; Dewangan *et al.,* 2013) and fish swim bladder (Kumar *et al.,* 2013b; Remya *et al.,* 2014; Kumar *et al.,* 2015). Biological ionic detergents like sodium deoxycholate are more effective in removing cellular remnants than sodium dodecyl sulfate (Gilbert *et al.,* 2006). Protocol for preparing the acellular bubaline diaphragm matrix was optimized by Kaarthick *et al.* (2017). Acellular diaphragm matrix (ADiaM) for the umbilical hernioplasty in cow calves has been reported where the hernial ring size ranged from 8-10 cm in diameter and found to be a promising and immuno-tolerable prosthetic material (Remya *et al.,* 2013; Shakya *et al.,* 2013; Remya *et al.,* 2014). Acellular diaphragm matrix was safely used in umbilical hernioplasty in pigs (Kumar *et al.,* 2015).

Fore-stomach matrix (FM) scaffolds have several advantages over existing tissue scaffolds and have various clinical applications. FM scaffolds can be formatted in a single or multiple sheets (Ward *et al.,* 2014). The fore-stomach has a higher trans-mural osmotic flow across the tissue layers facilitating separation of tissues layers and decrease in the time of decellularization. Fore-stomach matrix scaffolds have a higher biaxial strength as compared to other scaffolds (Ward *et al.,* 2014). The protocol for decellularization of the caprine rumen and bubaline diaphragm has been standardized in our laboratory. Acellular matrices derived from the bubaline diaphragm and caprine rumen were used for hernia repair in clinical cases. Hence, the objectives of the study were to test the acellular bubaline diaphragm matrix (BDiaM) and acellular caprine rumen matrix (CRuM) for the repaired hernia in clinical cases

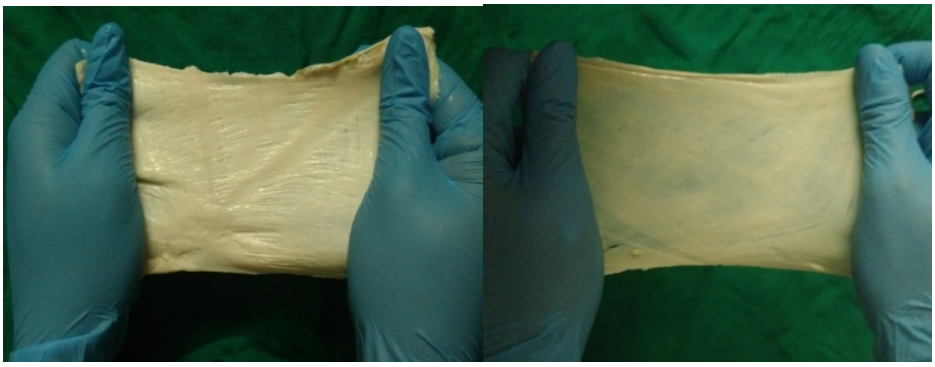
MATERIALS AND METHODS

Preparation of Acellular Matrices from Bubaline Diaphragm and Caprine Rumen

Fresh bubaline diaphragm (BD) and caprine rumen (CR) tissues were collected and kept in a saline solution containing 0.02% EDTA and 0.1% Amikacin and were washed few times with PBS having 7.4 pH. Tendinous part of the diaphragm was used for the preparation of the acellular matrix. The inner keratinized layer of caprine rumen was peeled off, and the serosal layer was removed. The tissue matrices were cut into pieces of the desired size and were subjected to decellularization protocols. Sodium deoxycholate (1% for CR and 2% for BD) was used at room temperature under physical agitation (180 rpm) on an orbital shaker for 48 h. Tissues were thoroughly washed with PBS and treated with absolute ethanol for four hours, then washed with PBS and stored at -20°C before clinical use. The degree of decellularization of the BD and CR was evaluated by histological examination and DNA contents analysis. Before and after decellularization protocol, approximately 5x5 mm2 sizes of tissue samples were cut and fixed in 10% formaldehyde in PBS. The sections were stained with hematoxylin-eosin stain. DNA was extracted using DNA isolation kit for mammalian tissues (Thermo Scientific, USA) and quantified as per method reported by Pellegata *et al.* (2013).

Clinical Application of the Acellular Matrices

The prepared acellular bubaline diaphragm matrix (ADiaM) was applied in 8 clinical cases (Group I), and caprine acellular rumen matrix (CRuM) was applied in 9 clinical cases (Group II) of hernias/abdominal wall defects. The cases having a hernial ring of more than 4 cm in diameter were included in the study. A thorough clinical examination was performed to determine the type of hernia, ring size, adhesions, etc. Standard anesthetic protocols were used for anesthetizing the animals. Pre-operative observations included signalment, etiology, prior treatment given (if any). The animals were positioned in dorsal posture or as per the location of abdominal wall defects. The hernia sac was exposed, and the contents were reduced, and the prepared acellular matrices (Figure 1a and Figure 1b) was used for closure of the hernial ring.



(a) (b)

Figure 1. (a, b): Prepared acellular matrices, a-Acellular diaphragm matrix, b-Acellular caprine rumen matrix

An appropriately sized acellular matrix was anchored with abdominal muscles with the underlying technique using the appropriate number of Polyamide suture (Sterilon, Stericat Gutstrings (P) Limited, Delhi, India). The subcutaneous tissue and skin were closed using the proper amount of Polyglactin 910 (Vicryl, Johnson and Johnson Limited, Ethicon, Aurangabad, India) and nylon suture, respectively. Postoperative analgesia was provided by an intramuscular injection of 0.5 mg/kg of Meloxicam (Intas Pharmaceuticals, Ahmedabad, India) for 3-5 days. Antibiotic Enrofloxacin (5 mg/kg IM) (Intas Pharmaceuticals, Ahmadabad, India) was given for five days. After removing skin sutures on completion of healing, all the animals were observed for three months for long-term evaluation of results.

Clinical Observations

Animals were observed for their feeding habits and general behavioral changes. The operated site was examined grossly till the completion of healing. Degree of swelling, degree of exudation, and degree of pain at the site were graded on 1-4 scales as per the method of Bigbie *et al.* (1991). Observations were recorded on day 0, 14, and 28 postoperatively.

Colour Digital Image Processing and Hematological Observation

Color photographs were taken before and after surgery at different time intervals and were used to evaluate the healing pattern. Differential leukocyte count (DLC) was done before the operation and on 14 and 28 postoperative days.

Immunological Observation

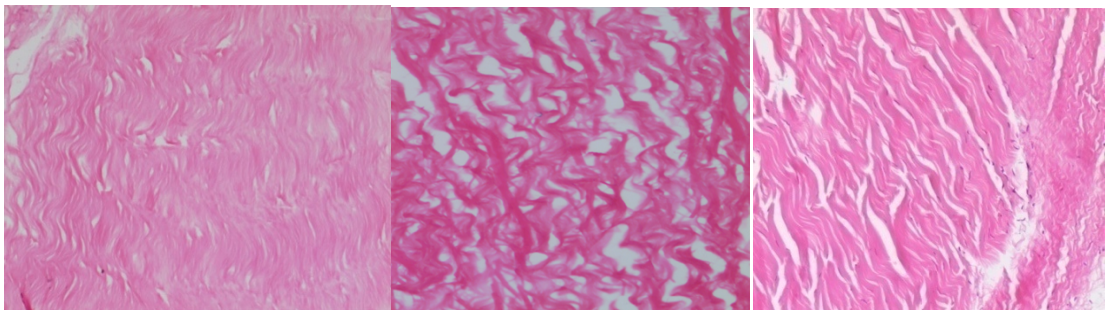
For immunological observations, ELISA was done following standard protocol. For this, serum was collected before implantation and on day 14 and 28 post-implantation. The serum protein distribution pattern on post-implantation days 14 and 28 were compared to day 0 by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Statistical Analysis

Statistical Program for Social Science (SPSS) was used to analyze the data. One-way analysis of variance (one-way ANOVA), Student's paired t-test, and Kruskal Wallis test was used (Snedecor and Cochran, 1990).

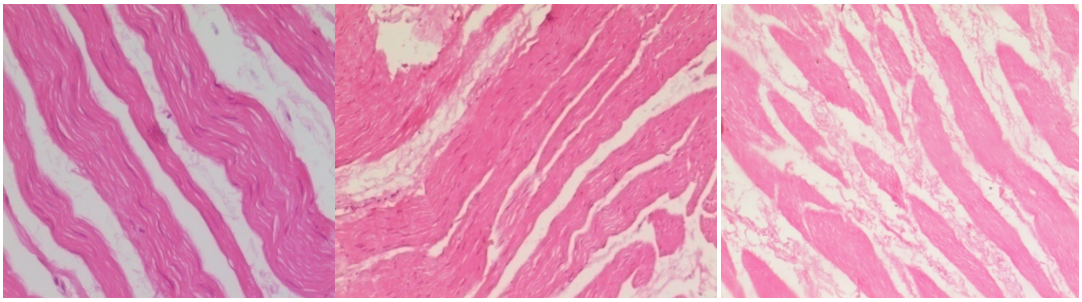
RESULTS

The native diaphragm before any treatment showed cellularity. Treatment with 2% concentration of SDC resulted in the loss of large numbers of cells at 24 h, while at 48 h complete acellularity was observed while preserving the distinctive, natural, three-dimensional collagen structures in the prepared matrix. The result of treatment with 2% SDC of the bubaline diaphragm is presented in Figure 2. Acellular matrix revealed the removal of cells and orderly arrangement of collagen fibers. Native rumen with no treatment showed cellularity. At 24 h of treatment with 1% SDC, decrease in cellularity of the ruminal tissue was observed. At 48 h of treatment, thin, loose arranged collagen fibers with very high porosity were observed. At 48 h of treatment thin, loose collagen fibers having high porosity and no cellular debris was seen. The result of treatment with 1% SDC of the caprine rumen is presented in Figure 3. Significant reduction in DNA content following decellularization was observed. The native bubaline diaphragm showed significant ($P<0.05$) reduction in DNA content from 11.23 ng/µl to 3.4 ng/µl and caprine rumen showed significant ($P<0.05$) reduction in DNA content from 130 ng/µl to 44.2 ng/µl. The result of DNA quantification is depicted in Figure 4.



Native 24h 48h

Figure 2. Bubaline diaphragm treated with 2% SDC at 24 and 48 h (H&E stain, X100).



Native 24h 48h

Figure 3. Caprine rumen treated with 1% SDC at 24 and 48 h (H&E stain, X100).

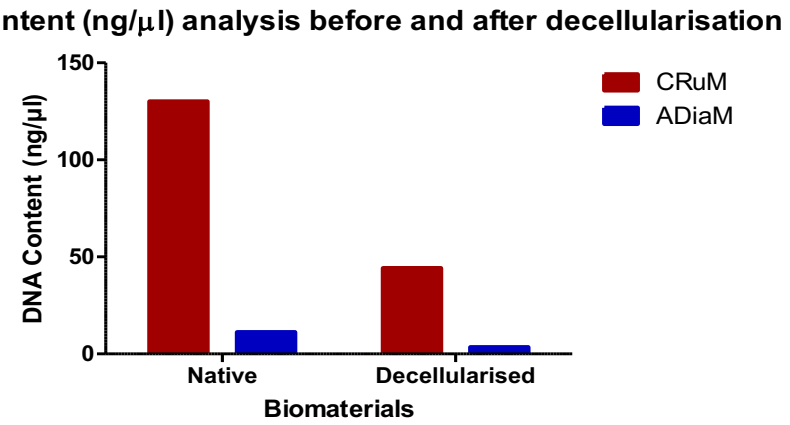


Figure 4. DNA contents (ng/µl) analysis before and after decellularization

Clinical Application of theAcellular Matrices and Observations

In this part of the study, acellular diaphragm matrix (ADiaM) and acellular caprine rumen matrix (CRuM)were used to reconstruct the abdominal wall defects in different animals. The detail of each case treated in both groups is described in Table 3. The site of hernia repair was examined up to 28 postoperative days. Mild to moderate temperature was observed during the first three days in both the groups that persisted up to the first week. All the animals remained dull and showed inappetence during the first 2-3 days and became normal within 5-7 days. Mean scores for the degree of swelling were calculated. On day 14, swelling scores had a non-significant difference between the two groups. Postoperative pain was observed up to day 7 in both the groups and exudation scores showed the non-significant difference between groups.

Table 3. Showing repair of abdominal wall defects (hernia) cases into both groups

S. No.	Species	Age	Sex	Diameter	Type of hernia	Hernial contents	Outcome
Group I: Acellular diaphragm matrix (ADiaM)							
1	Bovine	3M	M	6 cm	Umbilical	Large intestine	Repaired
2	Bovine	6M	F	8 cm	Lateral	Small intestine	Repaired
3	Bubaline	5M	M	5 cm	Umbilical	Omentum/omentalfat	Repaired
4	Bubaline	12M	M	10 cm	Ventral	Small intestine/mesentery	Repaired
5	Caprine	5M	M	8 cm	Ventral	Small intestine/mesentery	Repaired
6	Caprine	2M	F	14 cm	Ventral	Small intestine/mesentery	Repaired
7	Caprine	6M	F	7 cm	Lateral	I Intestine/omentum	Repaired
8	Swine	3M	M	6 cm	Umbilical	Omentum/fat	Repaired
Group II: Acellular caprine rumen matrix (CRuM)							
1	Bovine	3M	M	8 cm	Umbilical	Small intestine/mesentery	Repaired
2	Bovine	13Y	F	15 cm	Lateral	Uterus/foetus	Repaired
3	Caprine	4M	M	6 cm	Umbilical	Small intestine/mesentery	Repaired
4	Caprine	1M	M	14 cm	Ventral	Small intestine/mesentery	Repaired
5	Swine	3M	M	5 cm	Umbilical	Small intestine/mesentery	Repaired
6	Swine	3M	M	5 cm	Umbilical	Small intestine/mesentery	Repaired
7	Canine	4M	M	7 cm	Lateral	Small intestine/mesentery	Repaired
8	Canine	7Y	F	8 cm	Lateral	Small intestine	Repaired
9	Canine	1M	M	5 cm	Inguinal	Mesentery	Repaired

Colour Digital Image Processing and Hematological observations

Pre and post-operative digital photographs of both groups are presented in Figure 5 and Figure 6. The differential leucocyte count percent values (Mean ± SE) showed significant ($p<0.01$) decrease in neutrophils on day 14 and slightly elevated values ($p>0.05$) were recorded on day 28 postoperatively. A significant ($p<0.05$) increase in lymphocyte count was observed up to day 14 and returned to baseline values on day 28. No significant ($p>0.05$) change was observed in the eosinophils, basophils, and monocytes count.

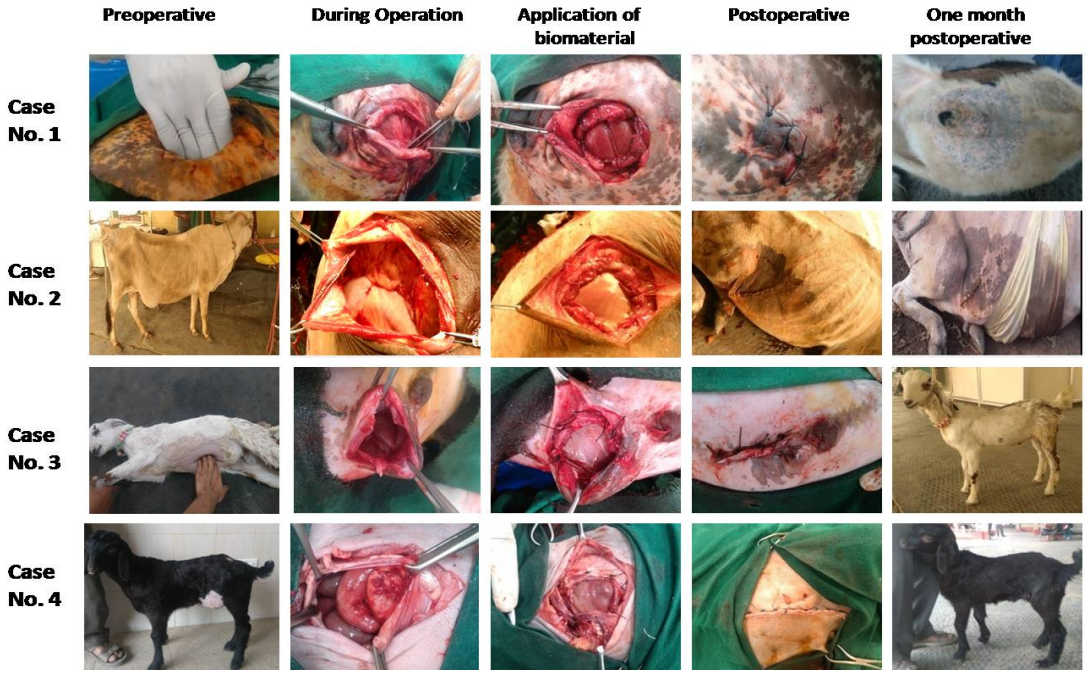


Figure 5. Gross observations at different time intervals in animals of group I (ADiaM)

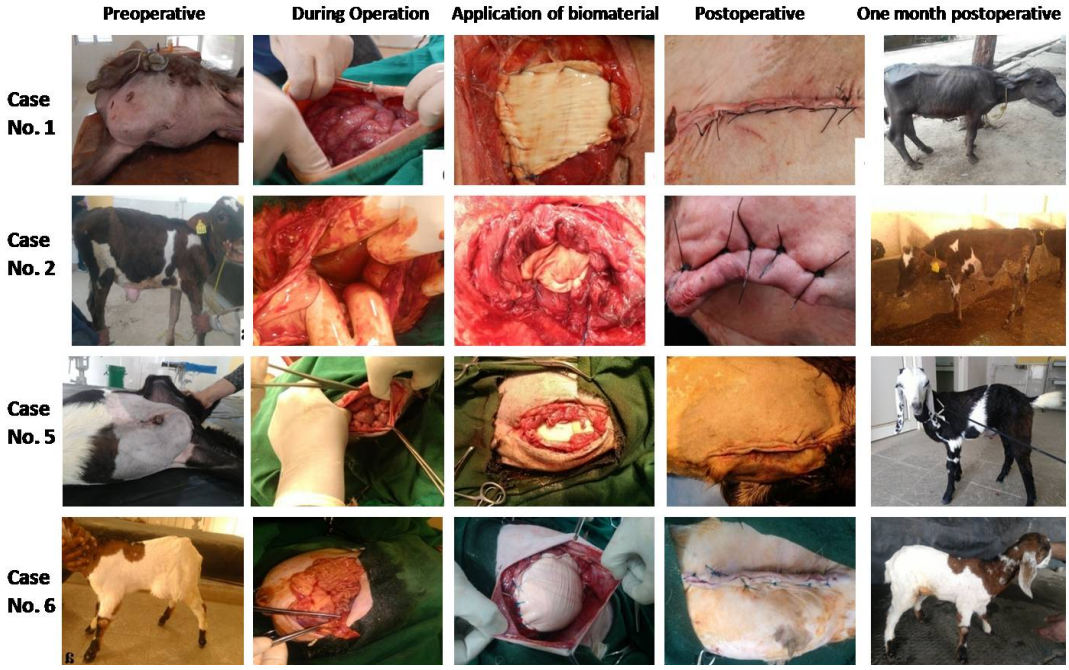


Figure 6. Gross observations at different time intervals in animals of group II (CRuM)

Immunological Observations

The results of the humoral immune response (intensity of color reaction as optical density) are presented in Figure 7. The level of antibodies in serum on day 0 was recorded as a base value. The sera collected on days 0, 14, and 28 from different implanted groups were evaluated for antibody titer. Antibody dilution of 1:200 was used throughout the experiment. On day 0 (before implantation), baseline OD450 nm values from acellular implanted groups showed no significant difference ($p>0.05$) from each other. Serum protein distribution became normal on day 28(Figure 8a and Figure8b).

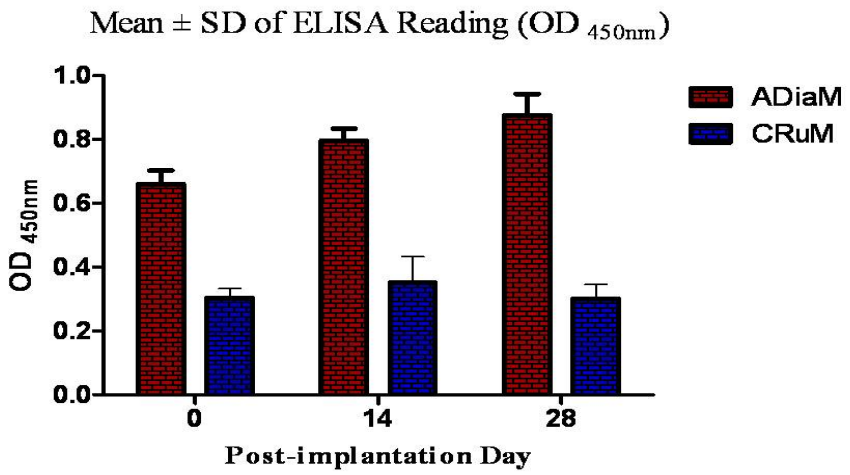


Figure 7. Mean ± SD of ELISA reading OD 450nm of two groups (I and II) in response to native tissue at different time intervals

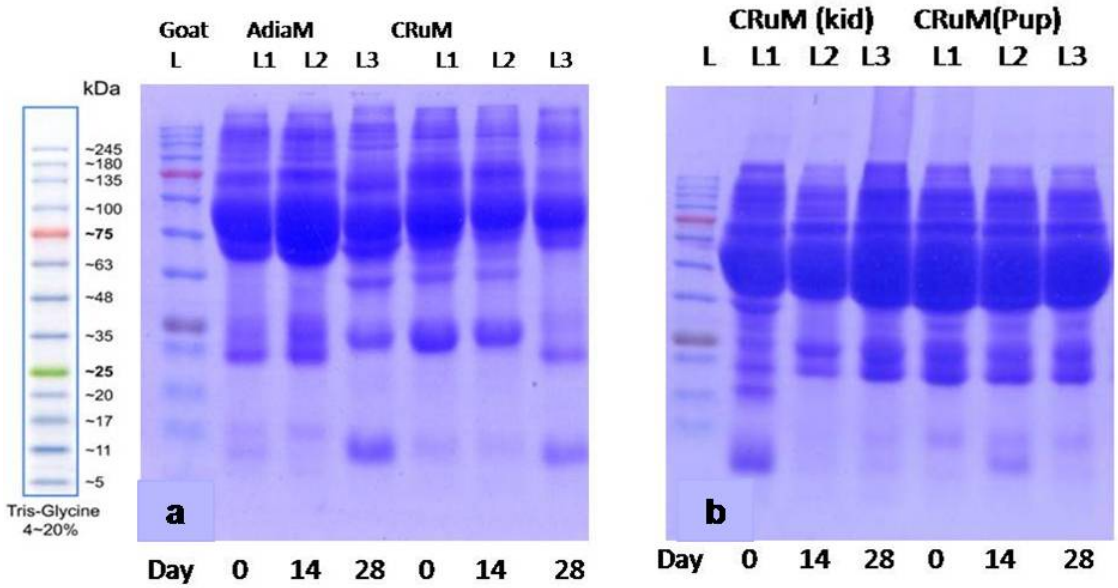


Figure 8. (a) SDS-PAGE at different time intervals of caprine species after application of ADiaM and CRuM; (b). SDS-PAGE at different time intervals after application of CRuM in a kid and pup.

DISCUSSION

Reconstructive surgery is the only method to restore the integrity of the abdominal wall and prevent incarceration and strangulation of herniated contents (Ober *et al.,* 2008). Hernial ring size exceeding 3 cm in diameter requires the use of prosthetic material for hernioplasty (Vilar *et al.,* 2009). Decellularized tissues have been used in preclinical animal trials and human clinical applications (Schmidt and Baier, 2000). Decellularization protocols also affect the biochemical composition, tissue architecture, and mechanical behavior of ECM (Gilbert *et al.,* 2006; Courtman *et al.,* 1994). Collagen derived from bovine or porcine was used in tissue engineering products. However, we used caprine collagen as a domestic goat available in the subcontinent as a potential source of collagen.

In the present study, bubaline diaphragm and caprine rumen were used as a biological material for reconstruction of abdominal wall defects because of ease of availability, ease of processing, and having sufficient tensile strength. Bubaline diaphragm incubated in 2 %SDC for 48 h at 37°C temperature under physical agitation was most effective for decellularization, with retention of the distinctive, natural configuration of collagen structures within the prepared matrix. Similar results were reported where bubaline diaphragm treated with 2% SDC for 48 h at 37°C was used in umbilical hernioplasty in pigs (Kumar *et al.,* 2015; Raghuvanshi *et al.,* 2018) and for perineal hernioplasty in dogs (Raghuvanshi *et al.,* 2018). Kaarthick *et al.* (2017) also documented that 1% SDC for 48 h resulted in the complete removal of cells and nuclear contents from the native bubaline diaphragm.

Goat rumen decellularized using 1% SDC for 48h resulted into the retention of the three-dimensional collagen structure. Acellular bubaline rumen matrix was decellularized using 1% SDC and used for reconstruction of umbilical hernioplasty in goats (Gangwar *et al.,* 2004) and buffalo calves (Singh *et al.,* 2018). The results of this study also support the notion that SDC is a viable option for decellularization of the bubaline diaphragm and caprine rumen. Umbilical hernia in cattle and buffalo constitutes more than half of all cases followed by ventral and lateral abdominal hernias. Umbilical hernia in the equines and bovines were found to constitute more than half of all cases in those species, followed by scrotal, ventral, or abdominally induced hernias (Farquharson, 1946).In the present study, mostly hernia cases were recorded in young age as compared to older age (Rings, 1995). Epidural anesthesia was administered followed by local infiltration analgesia. The animals were sedated using 0.1 mg/kg Xylazine HCl and were controlled in lateral or dorsal recumbency. No complication was observed in any of the animals due to anesthesia. Implantation techniques of prosthetic materials play important roles in hernia recurrence (Schumpelick *et al.,* 2004; Abouelnasr *et al.,* 2014).

Normal abdominal wall integrity without any postoperative complications was observed for three months postoperatively. Normal healing without hernia recurrence and postoperative complications or rejection of acellular matrix were seen in both groups as also reported using bubaline diaphragm (Kumar *et al.,* 2015) and bubaline rumen (Singh *et al.,* 2018). The decellularized collagen matrices support cell growth resulted in functional tissue regeneration (Badylak, 2004; Kumar *et al.,* 2015; Singh *et al.,* 2018). Animals showed normal behavior and growth rate during the follow-up period. The increase in temperature may be attributed to the action of endogenous pyrogen. Mild exudation at the operated site might be due to the acute inflammatory reaction in response to the surgical trauma. As the inflammation subsided gradually, no exudation was observed on day five onwards. Mild s pain was satisfactorily managed with non-steroidal anti-inflammatory drugs. Complete resolution of edema was seen between 7 and 12 days after surgery as also observed in pigs (Kumar *et al.,* 2015) and calves (Singh *et al.,* 2018).

In the present study, the differential leukocyte count revealed a significant ($p<0.05$) decrease in neutrophil percent on post-operative day14 as compared to day 0 and gradual return nearer to normalcy on day 28. This is plausibly due to acute inflammation whereas, a significant ($p<0.05$) increase in lymphocyte percent on post-operative day14 with gradual return nearer to normalcy on day 28. In indirect ELISA the absorbance values on day 0 were taken as a base value. Both the groups exhibited a temporal variation in humoral immune response from day 0 and subsequent follow-up day. The antibody titer in response to native tissue antigen increased on a postoperative day 14. Increase in antibody titer from day 0 to 45 after implantation of the acellular dermal matrix in rats was reported by Mohsina *et al.* (2013) and Karthick *et al.* (2017). SDS-PAGE analysis of the serum of both groups showed changed protein distribution patterns on day 14 as compared to day 0 and 28. These results were in accordance with observations of Kumar *et al.* (2015) and Singh *et al.* (2018). This may be attributed to slight inflammatory reactions in response to surgical trauma and the acellular matrices implantation.

Different biological-based biomaterials have been used for the reconstruction of abdominal wall defects/hernia in different species of animals. Raghuvansi *et al.* (2018) reported the clinical application of the extracellular matrix of animal origin in hernioplasty of different species of animals. The matrices included the bubaline aorta, rumen, diaphragm and caprine rumen. Use of bubaline and caprine rumen has been reported for abdominal wall reconstruction in different species of animals (Raghuvanshi *et al.,* 2018). Rathore *et al.* (2018) successfully used the bubaline acellular diaphragm matrix for the repair of abdominal wall defects in four different species of animals. Kumar *et al.* (2016) and Mathew *et al.* (2017) reported the use of rat dermal matrix as a biological mesh for repair of different hernias in dogs. Devarathnam *et al.* (2014) reported excellent biocompatibility of acellular aortic matrix of bubaline origin. Acellular aortic matrix for hernia repair has been used in dogs (Raghuvanshi *et al.,* 2018), buffaloes (Kumar *et al.,* 2012) and cow calves (Kumar *et al.,* 2013).

CONCLUSION

It is concluded that none of the animals showed recurrence, and smooth healing was observed. Both matrices viz. acellular bubaline diaphragm (ADiaM) and acellular caprine rumen (CRuM) were found biocompatible.

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