Indian Journal of Animal Sciences **86** (12): 1391–1395, December 2016/Article

Development and clinical application of decellularized porcine SIS and cornea for the repair of corneal defects in animals

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Received: 8 April 2016; Accepted: 23 May 2016

ABSTRACT

Porcine small intestine sub-mucosa (SIS) and cornea were decellularized using ionic biological detergent (1% SDS) and stored at –20°C in sterile phosphate buffer saline (PBS) solution containing mixture of antibiotics. The prepared biomaterials were subjected to histological and scanning electron microscopic observations to ascertain the decellularization status before their clinical application. Both the biomaterials were evaluated for the repair of corneal defects in 11 animals. Five animals having corneal defects repaired with SIS and 4 repaired with cornea demonstrated successful healing without clinical signs of infections or reoccurrence during the 8 months follow up period. Results of study support the use of decellularized porcine SIS and cornea as an alternative to traditional implantation materials to treat different corneal defects in animals.

Key words: Corneal defects, Cow, Decellularized, Dogs, Porcine cornea, Porcine small intestine sub-mucosa

Loss of vision due to corneal diseases is a major cause of blindness worldwide (Shin *et al.* 2003). Corneal diseases can occur due to mechanisms, which disrupt the normal corneal architecture, leading to blood vessel migration, pigmentation, odema, opacity, ulceration and loss of clear vision (Morreale 2003). Corneal surgery is essential for treatment of disorders of cornea not amenable to medicinal therapy alone, which varies from simple linear keratotomy for indolent ulcers to penetrating keratoplasty for restoration of optical clarity. Many corneal diseases are treated by corneal transplantation (Heindl *et al.* 2013). Mechanical devices and artificial prosthesis that are currently in use are not intended to integrate into the host tissue. Long-term wearing could induce inflammatory response in the host (Chapekar 2000). Many tissue engineered biomaterials like acellular amniotic membrane (AM), acellular porcine small intestinal submucosa (SIS) and acellular porcine cornea has been considered as a potential for the repair of coneal grafting in animals.

Small intestinal submucosa (SIS) has stimulated research investigation and clinical interest in recent years. SIS contains natural growth factors including basic fibroblasts growth factor (basic FGF) and transforming growth factor beta (TGF-beta). It has good tensile properties, non-immunogenic and resistant to infection, and when used as a xenograft promotes wound healing by providing a scaffold for tissue in growth (Griguer *et al*. 2001).

Frozen corneal grafts are primarily a source of collagen and serve a tectonic and therapeutic function in the absence of fresh tissue. If banked or fresh homologous cornea is not available, a lamellar corneal graft harvested from a healthy aspect of the recipient's cornea can be used (Denis 2004). Tissue-engineered cornea is being considered as a viable alternative to allogeneic corneas because of the shortage of graft materials. Acceptable level of corneal transparency was regained after transplant with a porous acellular corneal scaffold of porcine origin fabricated for effective pore size (Xiao *et al.* 2011).

The present study was therefore conducted to develop ready to use decellularized xenografts of porcine small intestine sub mucosa and porcine cornea and their clinical evaluation for corneal defect repair in animals.

MATERIALS AND METHODS

The study was conducted in the Division of Surgery, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly from June 2012 to May 2015 in two phases.

Phase 1. Preparation of decellularized biomaterials.

Phase 2. Clinical evaluation of decellularized biomaterials for the repair of deep corneal defects (Ulcers) in animals.

Phase 1. Preparation of decellularized biomaterials

Porcine small intestine and whole eye balls collected from local abattoir and institutional slaughterhouse were cleaned thoroughly by sterile physiological normal saline.

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The small intestine was cleaned thoroughly. The serosa and muscular layers were removed by scrapping. The small intestine was cut into 1×1 cm^2 pieces and placed in sterile PBS containing cocktail of antibiotics (penicillin, streptomycin and amphotericin). Similarly, cornea was separated and placed in sterile PBS containing cocktail of antibiotics, which included penicillin, streptomycin and amphotericin. Both porcine SIS and cornea were made acellular by treating with 1% SDS in an orbital shaker for 12 h. The prepared acellular scaffolds were thoroughly washed with PBS solution and placed in 70% ethanol for 12 h. They were again washed thoroughly in PBS and stored at –20°C in PBS solution containing mixture of antibiotics. For histological examination, the samples were placed in 10% formaldehyde solution and for SEM examination the matrix was kept in 2% glutaraldehyde. Prepared matrix scaffolds of SIS and cornea were subjected to histological and scanning electron microscopic examination to ascertain their acellularity.

Phase 2. Clinical evaluation of decellularized biomaterials for the repair of deep corneal defects (ulcers) in animals

Eleven animals including 10 dogs and a cow presented with full thickness corneal ulcers and injury to the referral veterinary polyclinic of ICAR-IVRI, Izatnagar, Bareilly were included in the study. Decellularized SIS was used in 5 canines and a cow. Decellularized cornea was used in the other 5 canine patients. The records were maintained for signalment, description and size of the ocular lesion (when available), postoperative complications, postoperative medical treatments, and any other intraoperative information generated.

Outcome in cases was recorded for corneal integrity and healing, synechiae and pupillary light reflexes. Vision was evaluated by the menace response. Full-thickness lacerations were repaired with decellularized SIS (6) and decellularized cornea (5). The standard procedure for these 11 cases included debridement (via keratectomy) of the necrotic and collagenolytic corneal tissue as well as excision of necrotic iridal tissue if present. For fixation of graft with simple interrupted 4 cardinal sutures were placed into the recipient site and a simple interrupted suture pattern using 8/0 or 9/0 in dogs and 6/0 in cow polyglactin 910 was used to secure the graft to the cornea. The suture material sizes were 8/0 or 9/0 in dogs and 6/0 in cow. Cases for each biomaterial were selected randomly. All cases were treated postoperatively with topical and systemic antibiotics and topical atropine. In cases of dogs, application of Elizabethan collar was advised to the owners to avoid self-inflicting injuries. Corneal dermoid was treated in one cow using a penetrating keratoplasty (PK) and placement of SIS into the corneal defect. The PK was performed using a #64 Beaver blade due to the large size of the dermoid. The excised dermoid tissue was submitted for histopathologic evaluation. A SIS graft was sutured into the defect in a manner similar to that utilized for the corneal perforations as described subsequently. Weekly follow-up and tarsoraphy sutures were removed 14 days post-surgery in all the cases.

RESULTS AND DISCUSSION

Macroscopic, histological and scanning electron microscopic examination of scaffolds: Macroscopic observation after decellularization revealed soft, spongy and glistening small intestinal submucosa (Fig. 1A). Histologically, native SIS showed the absence of lamina propria, tunica muscularis and loose connective tissue with blood vessels and lymphatics (Fig.1B). After decellularization, SEM observations of SIS revealed loss of cellular details with presence of acellular connective tissue mass (Fig.1C).

Macroscopic observation after 12 h of decellularization with 1% SDS revealed soft, opaque and turgid cornea with an increase in the thickness (Fig. 2A). Histological examination showed disruption of collagen fibres of stroma with presence of vacuolar spaces and absence of cells and nucleus (Fig. 2B). Scanning electron microscopy revealed loose collagen fibres and absence of cells in the decellularized scaffolds. The surface of decellularized cornea was smooth without presence of cells (Fig. 2C).

The results of the clinical cases repaired with SIS are summarized in Table 1. Five canine patients ageing 2 months to 6 year old (S No. 1–5) received SIS grafts to repair deep corneal ulcers/melting ulcer of different depth (Fig. 3A-C). Two of these had positive bacterial cultures. At the 2-week recheck examination of patient S.No. 1, the lateral edge of the graft was found dehisced. In none of the animals second surgery was done. Patient S No. 6 had a history of corneal dermoid that had been surgically debulked and it was treated by full-thickness surgical excision that resulted in a corneoscleral defect of 11 mm ×10 mm. SIS was sutured into the defect. Forty-five days post-surgery animal recovered uneventfully, no recurrence was reported by the owner (Fig. 5A-C).

Canine patients ageing 4 months to 7 year (S No 7–11) presented for a melting corneal ulcer, deep corneal ulcer, and corneal perforations (Table 1) were repaired with decellularized porcine cornea (Fig 4A-C). Patient serial number 7 was initially diagnosed with a 4-mm diameter deep corneal ulcer. Bacterial and fungal culture results were positive for *Staphylococcus aureus.* Bacterial culture of patient serial number 9 was also positive for *Streptococcus sp*. Patient serial number 10 had aqueous humor leakage 2 weeks after surgery but second surgery was not performed as the leakage stopped spontaneously and the anterior chamber reformed. The uveitis was also recorded in this case. Limited vision, and anterior synechia was observed in patient serial number 10 with a negative pupillary light response in the operated eye. In rest of four patients (serial number 7, 8, 9 and 11) with either deep or melting ulcer were visual postoperatively.

Decellularized tissues and organs were successfully used as scaffolds in a variety of tissue engineering/regenerative medicine applications and the decellularization methods

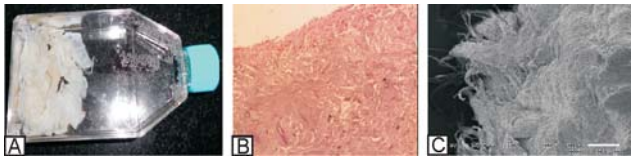

Fig.1 **(A-C)**. Gross appearance of decellularized porcine small intestine submucosa (**A**), Histology of decellularized porcine small intestine submucosa (H&E 20×) (**B**) and SEM image of decellularized porcine small intestine submucosa (**C**).

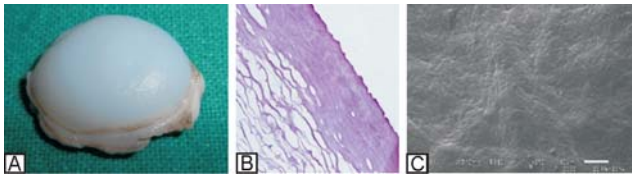

Fig. 2**(A-C)**. Gross appearance of decellularized porcine cornea **(A)**, histology of decellularized porcine cornea (20×) **(B)** and SEM image of decellularized porcine cornea **(C)**.

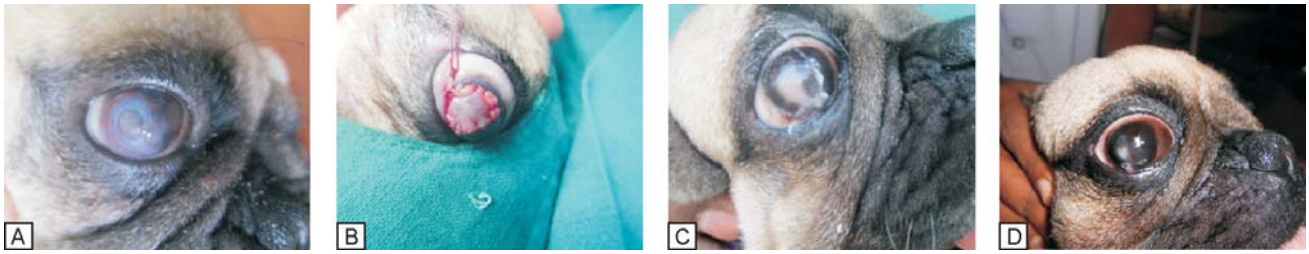

Fig. 3 **(A-D)**. Deep corneal ulcer in a dog (**A**) Repaired with decellularized porcine SIS; (**B**) 1month; (**C**) and 65 days; (**D**) Post surgery.

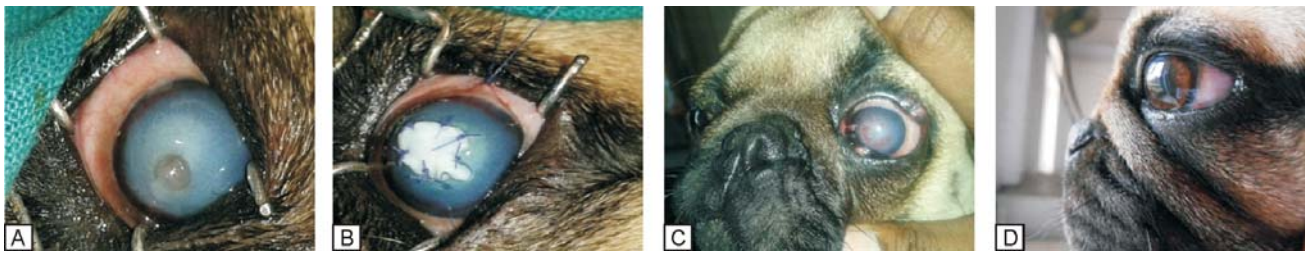

Fig. 4 **(A-D)**. Deep corneal ulcer in a dog **(A)** Repaired with decellularized porcine cornea; **(B)** 1 month; **(C)** and 85days; **(D)** Post surgery.

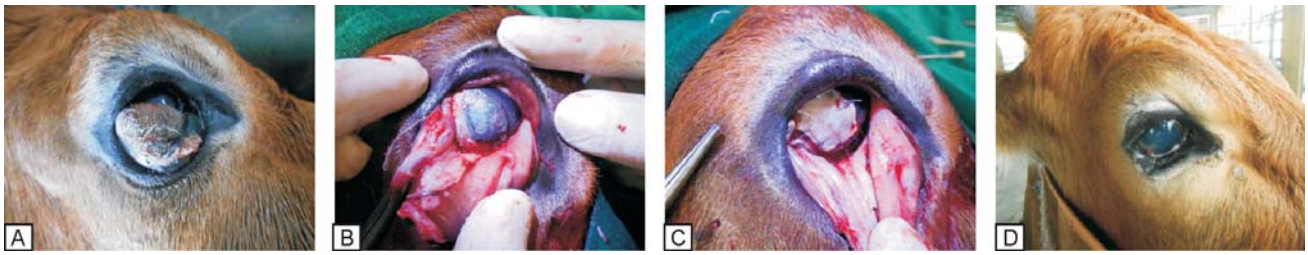

Fig. 5 **(A-D)**. Corneal dermoid cyst in a cow **(A)**; keratectomy for dermoid removal **(B)**; application of decellularized porcine SIS on the keratectomy defect **(C)**; animal 45 days post surgery complete healing of the keratectomy defect **(D)**.

varied widely as the tissues and organs of interest. Bio-scaffolds derived from decellularized tissues and organs were successfully used in both pre-clinical animal studies and in human clinical applications (Metcalf *et al.* 2002, Badylak 2004, Kolker *et al*. 2005). Removal of cells from a tissue or an organ leaves the complex mixture of structural and functional proteins that constitute the extracellular matrix (ECM). Several methods can be used to facilitate decellularization of tissue, including freezing and thaw, direct pressure and agitation and use of biological detergents.

In the present, study ionic biological detergent (1% SDS) was used for chemical decellularization. Histologically, loss of cellular details and nuclear remnants indicated an effective decellularization of the small intestinal submucosa. The fundamental ultrastructure of small intestinal submucosa appeared to be preserved in scanning electron microscopy. SDS is very effective for removal of cellular components from tissue. Compared to other detergents, SDS yield more complete removal of nuclear remnants and cytoplasmic protein such as vimentin (Woods and Gratzer 2005). SDS tends to disrupt the native tissue structure and cause a decrease in the glycosaminoglycan (GAG) concentration and a loss of collagen integrity (Gilbert *et al*. 2006).

The goal of a decellularization protocol is to efficiently remove all cellular and nuclear materials while minimizing any adverse effect on the composition, biological activity,

Table 1. Signalment and outcome of the cases treated with fill thickness decellularized porcine SIS and cornea

S No.	Breed	Age	Sex	Primary lession	Bacteriology	Complications	Outcome
Animals treated with decellularized porcine SIS							
1	Spitz	6 years	Male	Chronic ulcer	None	Graft dehisced	Blind severe pigmentation
2	Pug	4 months	Female	Melting ulcer	*E. coli* and *Pasteurella aerogenosa*	none	Successfully return of vision
3	Pug	6 months	Male	Chronic deep ulcer	None	none	Successfully return of vision
4	GSD	1.6 years	Male	Melting ulcer	*Staphylococcus epidermis*	none	Successfully return of vision
5	Pug	2 months	Male	Melting ulcer	None	none	Successfully return of vision
6	Cow	2.5 years	Female	Corneal dermoid	None	none	Successful
Animals treated with decellularized porcine cornea							
7	Pug	5 months	Female	Deep corneal ulcer	*Staphylococcus aureus*	none	Successfully return of vision
8	Pug	7 year	Male	Melting Ulcer	None	none	Visual
9	Pug	8 months	Female	Deep corneal lacerations	*Streptococcus* sp	none	Visual, corneal edema
10	Spitz	4 months	Male	Corneal perforation	None	Chronic uveitis and leakage of aqueous humor	Limited vision, anterior synechia
11	Pug	6 months	male	Deep ulcer	None	none	Visual

and mechanical integrity of the remaining ECM (Gilbert *et al.* 2006). Corneal thickness and hydration are associated with the function and number of corneal endothelial cell and corneal hydration has a linear relationship with corneal thickness (Zucker 1996). The swelling of cornea is a major cause of destruction of endothelial cell function after decellularization. Restoration of corneal transparency by immersion in glycerol was by taking over the pump function of corneal endothelial cells. But from our studies we observed that this effect was temporary and the cornea became hard and tough.

Various surgical techniques and graft materials have been used and studied for corneal defect repairs in animals (Hansen and Guandalini 1999). The present study reported the successful use of decellularized porcine SIS and cornea to repair full-thickness lesions such as corneal ulcers and corneal perforations in dogs and corneal dermoid in a cow. Five out of 6 animals that underwent corneal defects repair with SIS and 4 out of 5 repaired with cornea demonstrated successful healing without clinical signs of infections or reoccurrence. The success rate reported in this study is similar to that achieved with other graft techniques. Two reports on the use of frozen corneal grafts resulted in vision in 84 and 100% of dogs and cats, respectively (Hacker 1991). Whittaker *et al*. (1997) reported 100% of patients with vision following penetrating keratoplasty for deep corneal stromal abscesses in eight horses. Complications like aqueous leakage, conjunctival graft dehiscence and anterior synechia in these patients were observed. These complications were similar to those reported in other studies on corneal surgery in clinical patients (Featherstone *et al*. 2001). Aqueous leakage may be seen following inadequate number or inappropriate suture placement. Anterior synechia may occur secondary to aqueous humor leakage and/or pre or postsurgical uveitis. Anterior uveitis is the main cause of posterior synechia, cataract and fibrin formation (Peiffer *et al*. 1999). In all the patients of our study, tarsorraphy was done to provide more structural support and to control bacterial infections. The use of a corneal-scleral-conjunctival transposition to minimize the axial corneal opacity the healthy adjacent partial thickness cornea was utilized for the repair and no rejection was observed (Wilkie and Whittaker 1997). Compared to the use of SIS, the main limitation is that a corneal-scleral-conjunctival transposition cannot be used for large corneal defects because of the need for healthy surrounding cornea to serve as the autologous graft (Hansen and Guandalini 1999). Freshly harvested corneas are more commonly used

in human penetrating keratoplasty in order to obtain maximal corneal clarity. In veterinary medicine, fresh and frozen grafts are used, but the latter is more practical due to a readily available frozen corneal bank. In present study, decellularized porcine cornea was used with high success and having the advantage that it is a ready to use. Further it is also reported that transplantation of decellularized porcine cornea increases graft transparency and survival for longer periods as compared with fresh grafts (Lee *et al*. 2014).

These collagen-based lab prepared xenografts has the advantage of being cost effective, easily prepared and were easy to handle. When compared to fresh or frozen corneal grafts, decellularied SIS and cornea were very easy to store and can be used in desired size.

Growth factors known to influence tissue development and differentiation such as fibroblast growth factor (FGF-2) and transforming growth factor β (TGF-β) were identified in SIS and probably influence the mechanisms by which this biomaterial modulates wound healing and tissue remodeling (Voytik-Harbin *et al*. 1997). Small intestinal submucosa is capable of inducing host tissue proliferation, remodeling, and regeneration of tissue structures following implantation in the lower urinary tract, body wall, tendons, ligaments and blood vessels (Knapp *et al*. 1994). Small intestinal submucosa was reported to be resistant to persistent infection with *Staphylococcus aureus* in arterial autografts when compared to polytetrafluoroethylene arterial prostheses (Badylak *et al*. 1994). There are no studies that report how SIS tolerates infection in the cornea. Neovascularization of the cornea following implantation of SIS is probably due to the surgery, initial traumatic event, and to corneal repair rather than an immune rejection. Many publications report that SIS does not appear to stimulate cellular immune rejection in animal models (Prevel *et al.* 1995).

From our results it can be concluded that ionic biological detergent (1% SDS) produced an effective decellularization of porcine small intestinal submucosa and cornea. Lab prepared decellularized SIS and cornea were relatively inexpensive, ready to use and easy-to-handle biomaterials that appears to be suitable for the repair of full-thickness corneal defects in animals.

ACKNOWLEDGEMENT

The authors are highly thankful to the Director, ICAR-IVRI for the facilities provided**.**

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