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Evaluation of bio-engineered corneal scaffold for the repair of corneal defect in rabbit model

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ABSTRACT

Clinically healthy adult New Zealand white rabbits (27) of either sex, were randomly divided into three groups (A, B and C) having 9 animals each. Porcine cornea was made acellular by treating it with 1% sodium dodecyl sulphate (SDS). Rabbit bone marrow derived mesenchymal stem cells were seeded over this acellular matrix. A 5 mm diameter lamellar keratectomy wound was created over the peripheral cornea of rabbits in all the 3 groups. In group A, the corneal defect was managed by simple tarsorrhaphy without any graft and is treated as control. In group B, defect was repaired with decellularized porcine cornea and in group C, corneal defect was repaired with r-MSC seeded decellularized cornea. On the basis of clinical, pathological and scanning electron microscopic examinations, mesenchymal stem cell seeded corneal scaffold showed better healing and vision when compared to nonseeded scaffolds. Cell seeded corneal matrix was found to be an alternative to conventional means of surgical management of corneal ulcer.

Key words: Acellular, Cornea, Extracellular matrix, Keratectomy, Porcine, Rabbit

Ulcerative keratitis is the most frequently encountered ocular disease in small animal ophthalmology (Gilger *et al*. 2007). Ulcerative keratitis can be classified as superficial keratitis, deep corneal ulcer keratitis, descemetocele keratitis and perforation keratitis, with reference to loss of corneal layers (Kim *et al*. 2009). This can be treated with medications or using various surgical procedures depending on corneal stromal lesion severity (Choi *et al*. 2007, Kim *et al*. 2009). For the repair limited to the surface epithelium, collagen matrix (Orwin and Hubel 2000) and amniotic membrane (Tsai *et al*. 2015) are the extracellular based scaffolds that are used for reconstruction. Decellularization treatment largely preserve the native structure of stromal collagen with orthogonally arranged collagen fibrils, a textured appearance of Bowman's layer and the fiber-like structure of Descemets membrane (Hashimoto *et al*. 2010). The unique extracellular matrix organization of cornea provides sufficient mechanical properties in addition to innate biological cues which help to direct and organize cell function including differentiation, proliferation and migration.

The mesenchymal stem cells may prove useful for the repair and regeneration of a variety of mesenchymal tissues such as bone, muscle, marrow stroma and the cells produce useful growth factors and cytokines that may help in additional repair (Caplan 2006, Pittenger 2008). In addition to this enhanced regenerative potential, these cells can be exploited to ameliorate graft rejection hence to facilitate graft take (Le Blanc *et al*. 2003, Wang *et al*. 2013). The present study was carried out to evaluate the corneal wound healing potential of mesenchymal stem cell seeded acellular porcine cornea in rabbit model.

MATERIALS AND METHODS

This study was conducted with the approval of institutional animal ethics committee of the Indian Veterinary Research Institute.

Freshly collected porcine corneas were decellularized (Fig. 1a) as per standard protocol (Daniel *et al*. 2005). To confirm acellularity, histology was performed and native cornea (Fig. 1b) and decellularized cornea (Fig. 1c) were compared. Scanning electron microscopic examinations was carried out to evaluate surface morphology of decellularized scaffold (Fig. 1d).

Bone marrow was aspirated aseptically from the iliac crest of adult New Zealand white rabbit using a bone marrow aspiration needle under general anaesthesia with xylazine (xylaxin, Indian immunologicals limited, Hyderabad, India) @ 5mg/kg BW and ketamine (Ketamax, Troikaa Pharmaceuticals Limited, Dehradun, Uttarakhand,

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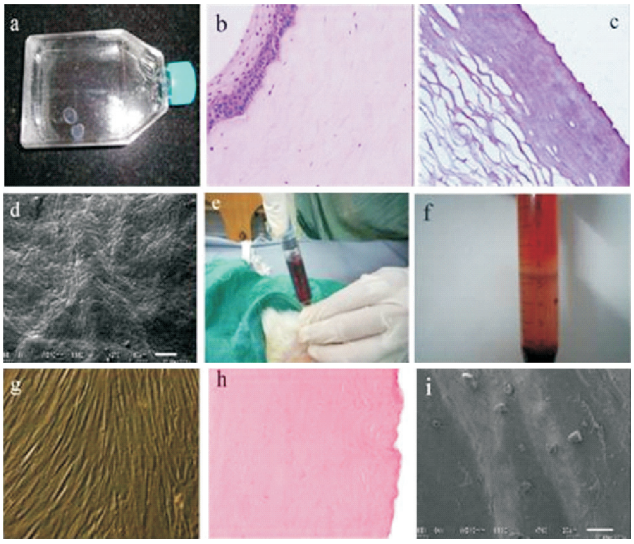

Fig. 1. a- Decellularized porcine cornea, b-Histology of native porcine cornea (H&E, 20×), c-Histology of decellularized porcine cornea (H&E, 20×), d-SEM of decellularized porcine cornea, e-Collection of BM from rabbit iliac crest, f-BM layered over density gradient medium, g-fully confluent r-MSC under phase contrast microscope without filter (20×), h-Histology of x-MSC seeded decellularized porcine cornea (H&E, 20×), i-SEM of x-MSC seeded decellularized cornea

India) @ 50 mg/kg BW (Fig. 1e). Isolation (Fig. 1f) and *in-vitro* culture of r-MSC were done with cell culture media DMEM inside biosafety cabinet as per standard protocol. The cell isolates were purified and maintained by subculturing upto third passage and were used for seeding purpose (Fig.1g). The cells were counted in a Neubauer counting chamber. Decellularized corneal scaffolds were kept in 6 well culture plate containing DMEM. The cells were seeded at a concentration of 2×10^4 cells/cm^2. Thereafter, the culture plates were kept in a CO_2 incubator maintained at 37°C in a humidified atmosphere of 5% CO_2. Morphological assessment of cell seeded matrix was done by histology (Fig.1h) and scanning electron microscopic examination (Fig. 1i).

The experiment was conducted in 27 clinically healthy adult New Zealand white rabbits of either sex. The animals

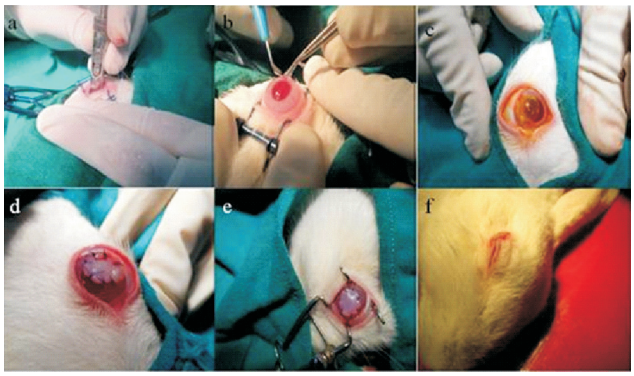

Fig. 2. a-Trephining of cornea, b-Lamellar keratectomy, c-fluorescein staining of the keratectomy wound, d-Defect repaired with decelluarized cornea, e-Defect repaired with r-MSC seeded decelluarized cornea, f-After tarsorrhapy.

Table 1. Treatment protocols given in different groups

Group	No of	Treatment
		animals
A	9	Without any grafting only tarsorrhaphy was done
B	9	Defect was repaired with decellularized porcine cornea along with tarsorrhaphy
C	9	Defect was repaired with r-MSC seeded decellularized cornea along with tarsorrhaphy

were acclimatized for 7 days in the new environment. Standard diet and water *ad lib.* were provided throughout the entire research period. The animals were randomly divided into three groups (A, B and C) of 9 animals each. In all groups, a 5 mm diameter corneal defect was made by trephining (Fig. 2a) and lamellar keratectomy (Fig. 2b) for assessing the healing potential of tissue engineered scaffolds. The defect was visualized by fluorescein dye staining (Fig. 2c). In group A, the corneal defect was managed by simple tarsorrhaphy and is treated as control. In group B, defect was repaired with decellularized porcine cornea (Fig. 2d) and in group C, corneal defect was repaired with r-MSC seeded decellularized cornea (Fig. 2e, Table 1).

Surgical procedure: Two days before the start of surgery, antibiotic drops were regularly instilled into the eyes. Animals were anaesthetized with xylazine (xylaxin, Indian immunologicals limited, Hyderabad, India) @ 5mg/kg and ketamine (Ketamax, Troikaa Pharmaceuticals Limited, Dehradun, Uttarakhand, India) @ 50mg/kg body weight given intramuscularly. The animals were restrained in lateral recumbency. The right eye was selected in all cases for surgical procedure. After trephining the cornea with a corneal trephine of 5 mm diameter, a partial thickness defect was created on the peripheral cornea in all the groups. In all groups, tarsorrhaphy was performed using nylon (Fig. 2f). Antibiotic (Enrofloxacin @ 5mg/kg body weight) and anti-inflammatory (Meloxicam @ 0.1mg/kg body weight) were given for 5 and 3 days postoperatively, respectively. antibiotic (Tobramycin), antifungal (Natamycin), and anti-inflammatory (Flurbiprofen) drops were instilled thrice daily for 2 weeks. Tarsorrhaphy sutures were removed on 15th postoperative day. The animals were observed for the evidence of corneal healing by subjective evaluation of cornea at 15, 30, 45 and 60 days postoperatively. After euthanizing the animals at 15, 30, 45 and 60 days, the healing was evaluated by histopathological and scanning electron microscopic studies. The efficacy of the bio-engineered scaffolds for the reconstruction of partial thickness corneal defect was evaluated on the basis of the following parameters.

Clinical observation

Corneal healing and vision: The healing status and gross appearance of corneal defects were examined by an independent observer after removal of tarsorrhaphy sutures

on 15th postoperative day, based on the following parameters and the examination was repeated on days 30, 45 and 60. *Conjunctival injection and chemosis*: It was scored as, 0 (absent), 1 (mild), 2 (moderate), 3 (severe).

Transparency of cornea: Transparency of cornea was scored as, 1 (totally clear); 2 (trace or faint corneal haze); 3 (mild haze of minimal density); 4 (moderately dense opacity partially obscuring inner ocular structure); 5 (severely dense opacity completely obscuring inner ocular structure).

Fluorescein staining of cornea: It was scored as, 0 (no dye uptake); 2 (< 25% staining of original wound); 3 (25~50% staining of original wound); 4 (50~75% staining of original wound); 5 (>75% staining of original wound).

Schirmer tear test: Schirmer tear test scoring was done as, normal (5 to 5.3 mm); mildly elevated (5.4 to 5.8mm); highly elevated (5.9 to 6.3 mm).

Pupillary light reflex and blink reflex: Scoring of the parameters was as follows: present (++); sluggish (+); absent (×).

Corneal neovascularization: It was scored as Grade 1 (no neovascularization); Grade 2 (maximum reach less than one-third the distance between the limbus and corneal centre); Grade 3 (maximum reach between one and two-third the distance between the limbus and corneal centre); Grade 4 (maximum reach more than two-third the distance between the limbus and corneal centre); Grade 5 (maximum invasion reaching the corneal centre).

Computerized planimetry: Colour photographs were taken and analyzed subjectively for healing status on days 15, 30, 45 and 60 postoperatively by an independent observer.

Histopathological observations: The cornea was collected from the animals sacrificed on days 15, 30, 45 and 60 postoperatively. The cornea was washed thoroughly in normal saline and fixed in 1% buffered formalin for 48 h and processed in routine manner for Haematoxylin and Eosin staining as per standard procedure. The stained sections were examined for the degree of cornel oedema, corneal thickening, leucocyte infiltration, neovascularization and fibroblast proliferation. Five high power microscopic fields were examined and mean score was taken to assess the healing status.

Neovascularization: Neovasularization was scored as: 1, resembling normal cornea (zero or one new blood vessel); 2, mild (two to five blood vessels); 3, moderate (six to ten blood vessels); 4, severe (greater than ten new blood vessels).

Corneal epithelial thickening: Corneal thickening was scored as 1 (normal); 2 (mild); 3 (moderate); 4 (severe).

Arrangement of epithelial cells: Arrangement of epithelial cells was scored as 1 (regular); 2 (irregular); 3 (highly irregular).

Fibroblast proliferation: Fibroblast proliferation was scored as 1 (nil); 2 (mild); 3 (moderate); 4 (severe).

Leucocyte infiltration: Leucocyte infiltration was scored as 1 (nil); 2 (mild); 3 (moderate); 4 (severe).

Scanning electron microscopic observations: The treated cornea was collected from the animals sacrificed on days 15, 30, 45 and 60 postoperatively. The cornea was washed thoroughly in normal saline and fixed in 2.5% gluteraldehyde in PBS overnight at 4°C and processed for scanning electron microscopy.

Statistical analysis: The data was analyzed by using The Statistical Programme for Social Sciences (SPSS). Two way analysis of variance (Two-way ANOVA) and Kruskal Wallis (One-way ANOVA) was used to compare the observations (Snedecor and Cochran 1989) and to determine the level of significance.

RESULTS AND DISCUSSION

A prompt and effective treatment strategy is essential to restore vision, to reduce pain (Kim *et al*. 2009) and immune-mediated rejection of the tissue in ulcerative keratitis. Decellularized tissues and organs have been used in a variety of tissue engineering or regenerative medicine applications (Conconi *et al*. 2005, Gilbert *et al*. 2006, Bolland *et al*. 2007). Biomaterial for corneal tissue engineering must demonstrate several critical features for potential utility *in-vivo*, including transparency, mechanical integrity, biocompatibility and slow biodegradation (Lawrence *et al*. 2009). Morphologically the undifferentiated rabbit MSCs are fibroblast like cells with spindle-shaped or polygonal cell bodies, similar to their rodent or human counterparts (Bourin *et al*. 2008). Similar morphology of r-MSC has also been reported (Lapis*et al*. 2008). Intramuscular administration of xylazine (5mg/kg body weight) and ketamine (50mg/ kg body weight) provided safe and satisfactory anaesthesia for creation of corneal defect and its subsequent reconstruction with different biomaterial scaffolds in rabbits. The same anaesthetic protocol was reported to be effective for the collection of bone marrow from the iliac crest of rabbits (Maiti *et al*. 2013).

During different observation days, the degree of conjunctival injection and chemosis was maximum in all groups by day 15, immediately after the removal of tarsorrhaphy sutures. This non specific response was likely due to the trauma of surgery since it is well known that the rabbit eye has a high sensitivity to the surgical trauma and rapidly responds by an inflammatory reaction, including conjunctival hyperemia, chemosis, iridial hyperemia, and miosis (Caprioli *et al*. 1985, Wickstrom 1991).

Optimal vision is contingent upon transparency of the cornea. The cornea is a highly complex sensory organ made largely of extracellular matrix components, developed and differentiated in a manner conferring optical transparency (Qazi *et al*. 2010). Corneal hazing that develops during the initial phase of corneal healing is due to irregularly oriented collagen fibers and accumulation of macromolecules such as proteins, glycosaminoglycans and lipids (Pedersen 2004). Maximum transparency of cornea was attained by 60th day of observation. In both the grafted groups, there was wound contraction in a centripetal fashion. The graft over the peripheral cornea initially created an opacity which gradually became clear. The mesenchymal stem cells seeded

porcine cornea grafted eyes attained much more clarity when compared to the nonseeded group. A healthy cornea has excellent self renewal activity, and in normal condition, damaged corneal epithelium is healed by reepithelialization (Kim *et al*. 2012). Because of this reason there was no visible scar tissue formation or scarring of cornea in the control group.

The water-soluble dye molecules diffuse into the intercellular spaces between living cells. Fluorescein is also used in identifying and monitoring corneal epithelial defects, corneal ulcers (Kumar and Thirumalesh 2013). Corneal epithelialisation was completed by 7 to 10 days. By day 15, the remnant of the graft on the cornea only had taken dye. There was no uptake of fluorescein dye in any of the treatment group by 30th postoperative day (Fig. 3).

The Schirmer tear test without topical anesthesia may be useful in rabbits for evaluation of increased values correlated with ocular irritation, rather than for determination of decreased values associated with keratoconjunctivitissicca (Abrams *et al*. 1990). The defect created over the cornea elevated the tear production. In grafted groups, in addition to the defect, the biological scaffold placed over the cornea might have acted as an extraneous material increasing the tear production. In all the groups there was elevated tear production initially which later on subsided to near normal values.

In the contol group the pupillary reflexes were normal. In addition to controlling the amount of light that enters the eye, the pupillary light reflex provides a useful diagnostic tool. It allows for testing the integrity of the sensory and motor functions of the eye (Purves *et al*. 2008). In both the grafted groups, the biomaterial placed on the cornea created a hindrance to the normal reach of light and threatening gesture, giving a reduced pupillary response. Once the graft absorbed and transparency regained, both the pupillary and menace reflex became normal.

Corneal epithelium is the most densely innervated epithelium in the body. The dense concentration of nerve endings in the epithelium accounts for severe pain observed with superficial epithelial loss, whereas a deep ulcer often does not exhibit the same degree of pain (Duke-Elder and Gloster 1968). Corneal surgery can cause corneal neurotrophic ulcers. These ulcers result from loss of the sensory innervation of the cornea, which leads to a decrease in the number of corneal stem cells, decreased metabolic and mitotic rates in the corneal epithelium (which increases cell permeability), and reduced acetylcholine and choline acetyltransferase concentrations (Mittag *et al*. 1974). There was a normal blink reflex in control group by 15 days suggesting complete epithelialisation of the cornea. In grafted groups once the grafts got completely resorbed normal blink reflex was observed.

In control group, the created superficial ulcer healed by epithelialisation without any stromal scarring. But in grafted groups, there was corneal neovascularsation and stromal scarring. In mesenchymal stem cells seeded group, there was reduced neovascularisation of cornea. The rate of corneal neovascularization coincides with other reported observations on corneal angiogenesis induced by xenografts in rats (Benelli *et al*. 1997) and biodegradable polymers in rabbits (Kobayashi *et al*. 1992).

Varying extent of implanted biomaterial remained over the cornea in grafted groups by day 15 (Fig. 4). Similar findings were reported by Griguer *et al*. (2001).There was complete resorption of the graft by day 30. Neovascularzation was evident over the grafted area. The

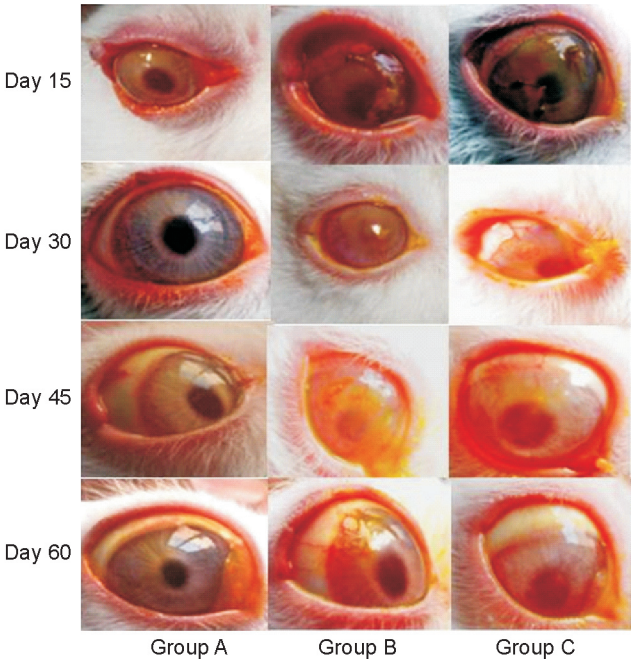

Fig. 3. Fluorescein staining of different treatment groups depicted according to observation interval.

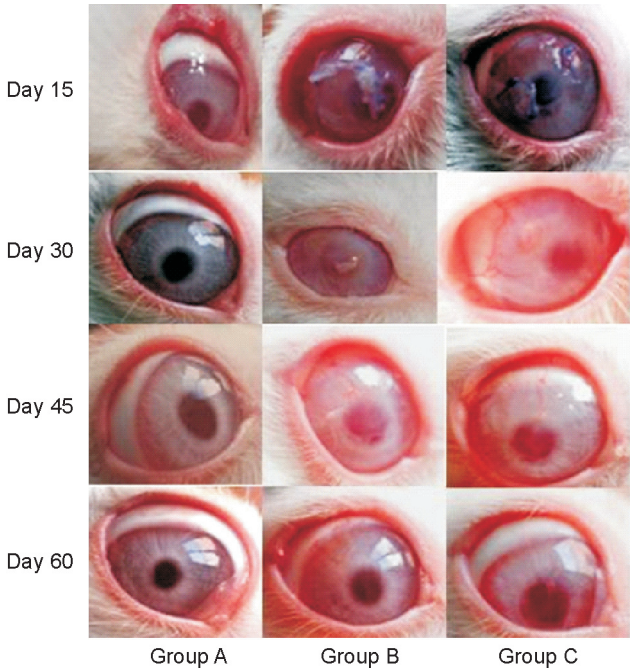

Fig. 4. Computerized planimetry of different treatment groups depicted according to observation interval.

newly formed blood vessels were superficial and they reached upto the centre of the cornea. Cornea started regaining clarity by day 45 and by day 60, the group C showed maximum clarity of cornea when compared to non-seeded group (Fig. 4). Corneal neovascularization also exhibited same trend. Four weeks post xenotransplantation into rabbit corneal stromal layers, a complete transparency was noticed and the corneas remained transparent with regular stromal structures and stable *in situ* without an immunological rejection up to one year after surgery (Yoeruek *et al.* 2012).

On histopathological observations in group A, by 15th day (Fig. 5a1) signs of epithelial regeneration were observed. The newly formed collagen fibers in stroma were loosely arranged. The nuclei of the anterior epithelium were hyperchromatic and there was neovascularization with presence of lymphocytes in the stroma. By 30th day (Fig. 5a2), stromal collagen fibers became more compact with much more lymphocytes, higher neovascularization and severe inflammatory reaction. By 45th day (Fig. 5a3), the extent of neovascularization was less and the stromal tissue seems to be immature when compared with normal tissue. By 60th day (Fig. 5a4), the stromal tissue appeared loose with neovascularization.

In group B, by 15th day (Fig. 5b1) complete regeneration of the lining epithelium with normal arrangement of cells was observed. Subepithelial stromal tissue revealed focal infiltration of lymphocytes, mild to moderate neovascularization and loosely arranged collagen fibres with moderate fibroblastic activity. By 30th day (Fig. 5b2), the anterior epithelium showed complete regeneration with mild increase in thickness. Subepithelial stromal tissue showed severe fibroblastic activity and severe lymphocytic reaction. Neovascular tissue, i.e. newly formed blood vessels were congested with focal hemorrhages. By 45th day (Fig. 5b3), the anterior epithelium appeared to be normal with little increase in thickness. Subepithelial stromal tissue showed presence of loosely arranged collagen fibers with occasional presence of lymphocytes, mild fibroblastic activity and presence of few newly formed blood capillaries. By 60th day (Fig. 5b4), anterior epithelium appeared normal with normal thickness and regular arrangement of cells. Subepithelial stromal tissue showed loose to dense connective tissue fibers with mild neovascularization and lymphocytic and fibroblastic reaction.

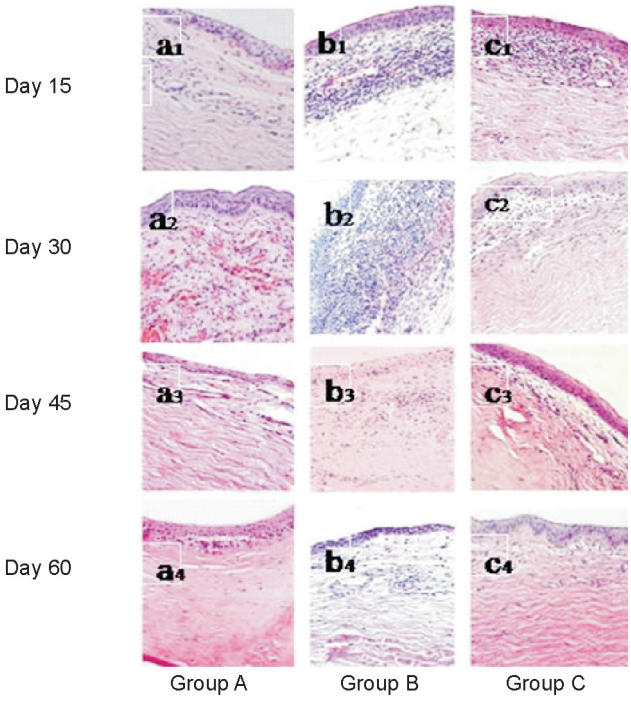

Fig. 5. Histopathology of the treatment groups depicted according to observation interval.

In group C, by day 15 (Fig. 5c1) the anterior epithelium revealed complete regeneration and was moderately thickened with regularly arranged cells. Subepithelial stromal tissue showed loosely arranged collagen fibers with moderate lymphocytic infiltration and fibroblastic activity. By day 30 (Fig. 5c2), the anterior epithelium appeared normal with regular arrangement of cells. Subepithelial stromal tissue appeared moderately dense with mild neovascularization and mild lymphocytic and fibroblastic activity. By day 45 (Fig. 5c3), anterior epithelium showed normal appearance with normal thickness. Subepithelial stromal tissue appeared moderately dense with mild neovascularisation and mild lymphocytic and fibroblastic activity. By day 60 (Fig. 5c4), the appearance of cornea was similar to that of normal cornea with moderately dense stromal collagen fibers. Histopathological examination revealed that among the two grafted groups, healing was better in group C where the scaffolds were seeded with bone marrow derived mesenchymal stem cells.

The biological materials generally combat infection, induce a mild inflammatory response, angiogenesis and host cell migration (Bellows *et al*. 2007). It can act as a regeneration template during wound healing. The first sign of graft acceptance is neovascularization of the graft bed. The frequent initiators of corneal neovascularization are hypoxia, neoplasia and inflammation. These events trigger angiogenic factors such as tumourangiogenic factor, fibroblast growth factor, transforming growth factor beta, platelet derived growth factor and vascular endothelial growth factor. Lymphokines produced by activated T-lymphocytes and prostaglandin (PG) E1 are capable of producing corneal neovascularization (Benezra *et al*. 1978, Glasers *et al*. 1980, Lutty *et al*. 1983).

Extent of corneal neovascularization was less in stem cell seeded group when compared to the non seeded group (Ma *et al*. 2006). MSC transplantation in the wounded cornea using direct application to the wounded corneal epithelium and systemic injection showed engraftment of transplanted MSCs in the cornea (Ye *et al*. 2006, Oh *et al*. 2008). The engrafted MSCs failed to transdifferentiate into corneal epithelial cells, and therapeutic effects of MSCs through paracrine effects of MSCs were suggested, which suppress inflammation and inhibit angiogenesis (Oh *et al*. 2008, Ma *et al*. 2006). It is also reported that tissue

engineered cornea analogs would provide effective cornea tissue substitutes and alternatives to address the need to reduce animal testing of commercial products (Ghezzi *et al*. 2015).

Histologically, the scar tissue was organised with keratocytes and collagen fibers with an overall sagittal orientation, parallel to the corneal surface. Since orientation of keratocytes reflects the direction of repair and the potential recovery of corneal integrity (Melles *et al*. 1994, Hashimoto *et al*. 2010), the regular arrangement of corneal fibroblasts in the scar tissue suggests that lamellar continuity across the wound might be obtained over time and result in a degree of stromal organisation close to normal. Implantation of thick acellular porcine corneal stoma with keratocytes could achieve a transparent cornea 6 months after the operation. However, very few cells were observed in the scaffolds even though the same amount of keratocytes were seeded during the operation (Ma *et al.* 2015).

Normal rabbit cornea showed a characteristic wavy pattern in scanning electron microscopy (Fig. 6). In higher magnification, polygonal cells and microvilli were also observed. In all the treatment groups, the corneal surface was highly irregular. Healing of the corneal stroma produced varying extend of scar tissue. The scar tissue was formed maximum by day 30 in all the treatment groups. The extent of scar tissue was much less in mesenchymal stem cells seeded scaffolds when compared to non-seeded scaffolds. Even by the end of 60 days, the healed cornea has not attained the appearance of normal cornea suggesting ongoing remodeling of corneal scar tissue.

The scanning electron microscope seems to bridge the gap between the usual histologic preparations and the biomicroscope because it provides a greatly magnified,

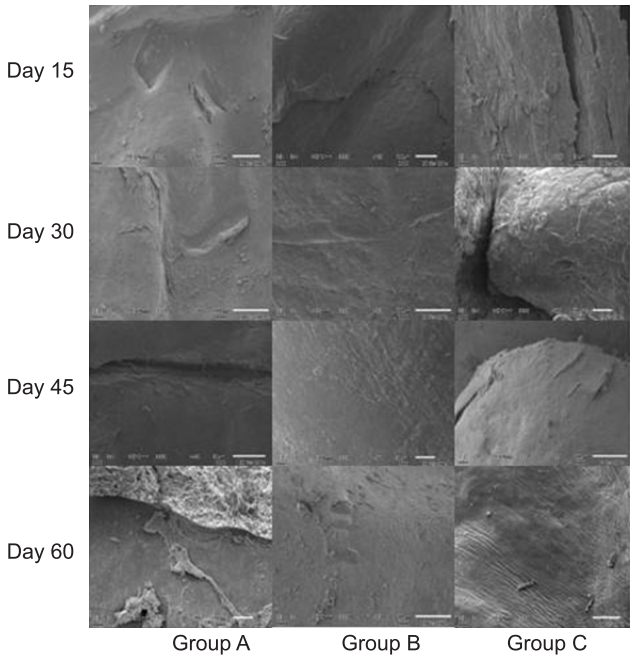

Fig. 6.Scanning electron microscopy of the treatment groups depicted according.

three-dimensional picture of the anterior (Spencer and Hayes 1970) and posterior corneal surfaces (Inomata *et al*. 1970). Scanning electron microscopic studies have shown that the superficial corneal epithelium is composed of fine ridges which are very sensitive to chemical and mechanical stimuli (Kuwabara 1970). In pathological conditions, the surface becomes irregular, pits appear, and the formation of cytoplasmic protrusions and separation of cells are common. The present study demonstrated an irregular surface of cornea in all the treatment groups due to the formation of granulation tissue subsequent to the healing of cornea. Pfister (1975) reported a study of epithelial abrasion in rabbits, using the scanning election microscopy. The immediate response to injury was separation and thickening of the basal and squamous epithelial cells at and near the margin of the wound. In the treatment groups, there was no evidence of line of rejection in any of the grafted group which is the sign of graft rejection (Polack 1972). The normal rabbit cornea showed a wavy pattern under scanning electron microscope. After lamellar corneal grafting, the healing of corneal surface generated scar tissue over the cornea which was not having a wavy pattern under scanning electron microscope (Cintron *et al*. 1982).

Our study demonstrated that treatment with 1% SDS for 12 h resulted in the complete acellularity of porcine cornea. Acellular corneal matrix aids in the healing of experimental corneal defects in rabbits. However, seeding of acellular corneal scaffold with rabbit MSC increases the healing potential by reducing corneal scarring and neovascularization.

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