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Stability of limbal stem cell deficiency after mechanical and thermal injuries in mice

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ABSTRACT

We studied the reproducibility and stability of limbal stem cell deficiency (LSCD) in mice following controlled injuries to the corneal and limbal epithelia. In one method, corneal and limbal epithelia were entirely removed with a 0.5 mm metal burr. In the other, limbus to limbus epithelial removal with the burr was followed by thermal injury to the limbus. These two methods were compared with a previously published one. Unwounded corneas were used as control. The corneas were examined monthly for three months by slit lamp with fluorescein staining. Immunofluorescence staining for cytokeratin 12 and 8 on corneal wholemount and cross sections were performed to determine the phenotype of the epithelium. Mechanical shaving of the epithelium, with or without thermal injury, resulted in a reproducible state of LSCD marked by superficial neovascularization, reduce of keratin 12 expression and presence of goblet cells on the cornea. The phenotype was stable in 100% of the eyes up to at least three months. Thermal injury produced a more severe phenotype with more significant stromal opacification. These corneal injury models may be useful for studying the mechanisms leading to limbal stem cell deficiency.

A clear cornea is essential for normal vision. Corneal epithelium, the most superficial layer of the cornea, protects it against pathogen invasion and is essential for maintaining the integrity and clarity of the cornea. The corneal epithelium is a stratified squamous epithelium that is endlessly renewed throughout life (Movahedan et al., 2013). The epithelial stem cells that maintain the corneal epithelium are primarily located at the corneal limbus in the basal layer of the epithelium (Ahmad et al., 2006; Dua et al., 2003). These stem cells are constantly self-renewing, repairing, and regenerating the corneal epithelium (Chang et al., 2008). After an injury to the epithelium, the remaining epithelial cells flatten, spread and move across the defect. The cells in the basal layer with proliferative capacity undergo further proliferation to restore cell numbers and cell mass. The newly regenerated epithelium is anchored firmly to the underlying tissue and the new basement membrane (Dua et al., 1994). This process is mediated by growth factors, adhesion proteins, proteases, cytokines, and other factors provided in part by the limbal blood supply, tear film, stromal and immune cells (Freire et al., 2014). Interactive cross-talk between surrounding cells, the extracellular matrix, and soluble signals are critical for epithelial homeostasis and the ocular surface wound healing response (Dua et al., 2003; Gipson, 1989).

Limbal Stem Cell Deficiency (LSCD) is a pathologic state that results from failure of stem cells to renew the corneal epithelium (Hatch and Dana, 2009; Sejpal et al., 2013). Inherited forms of LSCD, for example, are seen in aniridia (Mayer et al., 2003) and ectodermal dysplasia (Di Iorio et al., 2012) while acquired LSCD can arise following chemical injuries, Stevens Johnson syndrome and long-term contact lens wear (Chan and Holland, 2013; Huang and Tseng, 1991; Puangsricharern and Tseng, 1995). Because stem cells and/or their niche are lost or dysfunctional, corneal epithelial regeneration is impaired and corneal surface becomes repopulated by conjunctival epithelial cells ("conjunctivalization"), a finding that is considered the hallmark of LSCD (Dua et al., 2003). Conjunctivalization has clinical implications for patients. Most significantly, it causes superficial corneal neovascularization with recurrent/persistent epithelial defects, chronic ocular surface inflammation, scarring and loss of vision (Hatch and Dana, 2009;

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Puangsricharern and Tseng, 1995).

Understanding the pathophysiologic mechanisms of LSCD is critical to the development of novel therapies for this challenging condition. Animal models of LSCD are necessary both for studying the disease process at a cellular and molecular level and for testing the efficacy of treatments. Some of the earliest experimental models of LSCD were based on chemical destruction of the limbal area, particularly with alkaline agents (Luengo Gimeno et al., 2007; Phan et al., 1991). Alkali substances are known to cause severe ocular damage and inflammation with compromising stroma and resulting in corneal ulceration, hyphema, hypopyon, and even corneal perforation (Ma et al., 2006). Other models involve surgical removal of the limbal tissue (Chen and Tseng, 1991), cauterization (Majo et al., 2008) and more recently, using benzalkonium chloride to simulate chronic injury to the ocular surface (Lin et al., 2013). In this study, our goal was to evaluate and validate a reproducible mouse model of LSCD using mechanical and thermal injury in a controlled manner, thereby avoiding extensive damage to the corneal stroma and other anterior chamber structures.

All animal investigations were carried out in accordance with recommendations of Association for Research in Vision and Ophthalmology (ARVO). Experiments were performed on 84 eyes of four to six month-old male and female C57BL/6J mice. Eyes were clinically normal under slit lamp examination before conducting the experiments. Subjects were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) mixture. A drop of proparacaine 0.5% was instilled before and during the procedures. Three methods were conducted to generate LSCD: mechanical alone -using a blunt spatula or Alger Brush- and mechanical alongside with heat injury.

We have previously described a model of partial LSCD (Amirjamshidi et al., 2011), which was a modified mouse model described by Pal-Ghosh et al. (Pal-Ghosh et al., 2008). The model involved scraping the entire corneal epithelium from limbus to limbus using a blunt metal spatula with a 0.3 mm tip. The main advantages of that model are that it limits the injury to the epithelium and is able to produce a state of partial limbal stem cell deficiency. However, its main disadvantage is that the degree of limbal stem cell deficiency is variable since it is difficult to standardize the injury. In the current study, the blunt spatula method is compared with two others using AlgerBrush II rust ring remover (0.5 mm burr, Rumex international Co, Clearwater, FL) alone or followed by heat injury (16 eyes for each group). Unwounded corneas served as control.

Method one: Using Algerbrush II, the limbal epithelium was shaved twice after which, the entire corneal epithelium was removed once from limbus to limbus. Care was taken to avoid injuring the stroma and conjunctiva (Video 1). Brush tip was cleaned as required.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.exer.2015.11.012.

Method two: Limbal and corneal epithelium were removed with AlgerBrush II as described above in method one, followed by controlled thermal injury to the limbus. To exert a precise thermal injury, we designed an adjustable electric cautery that could yield a constant and measurable amount of heat at its fine tip. We used an ophthalmic cautery device (Medtronic Low Temp Cautery, Jacksonville, FL) connected to an adjustable electrical power supply. A 1.5 cm single solid copper wire (gauge 16, GLT, Solon, Ohio) was bent on itself and was tied to the tip of the cautery on the other end to create a very fine blunt tip (Video 2, epithelial removal not shown). The optimum temperature at the copper tip to create the desired damage without perforation was found to be $50 \pm 1\ ^{\circ}C$. The temperature was measured indirectly by measuring the temperature of 150 μL water heated with the cautery tip (the water was heated for at least 30 min and the temperature was recorded when the thermometer showed a constant value for five minutes). Limbal injury was induced by touching the instrument to the limbal area for two to three seconds -just long enough to cause a slight impression on the eyeball (Video 2).

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All corneas were examined by Nikon FS-2 photo-slit lamp under bright field and cobalt blue filter three minutes after applying 1 mg/ml fluorescein sodium (late fluorescein staining) on days zero, 30 (data not shown), 60 and 90 after the procedures. Fluorescein staining on day zero confirmed complete epithelial removal throughout the cornea and limbus (Fig. 1A). Histologic examination was used to confirm that the injuries did not violate the stroma (Fig. 1B), although keratocyte loss in the anterior stroma was seen as expected following an epithelial debridement (Wilson et al., 1996, Fig. 1B).

Platelet endothelial cell adhesion molecule-1 or CD31 was used as an endothelial cell marker. To confirm and visualize corneal vascularization, wholemount immunostaining for CD31 (anti-CD31 antibody, Biolegend inc., San Diego, CA, 1:50) was carried out on four corneas of each group three months following the injury (Fig. 1C).

Corneal epithelial phenotype was evaluated by immunostaining for cytokeratin (CK) 12, an intermediate filament specific to normal corneal epithelial cells (Liu et al., 1993) and CK8, a marker of simple epithelia (Pajoohesh-Ganji et al., 2012). Following euthanasia at three months, eyes (16 in each group) were enucleated and corneas were prepared for wholemount staining similar to what described previously (Amirjamshidi et al., 2011). Double indirect immunostaining of corneal wholemounts for CK12 (goat polyclonal anti-CK12, Santa Cruz, CA, 1:100) and CK8 (rat monoclonal anti-CK8 TROMA-I, Iowa City, IA, 1:50) were carried out (Fig. 2A). Cytokeratin-12 and CK8 immunofluorescence densities were separately measured in the central corneal of wholemount images using Image-J software (version 1.47, NIH) with correction for the background (Burgess et al., 2010).

Normal uninjured corneas demonstrated CK12 throughout the corneal epithelium. In both Algerbrush and combined AlgerBrush and thermal injury groups CK12 was almost totally absent at three months, while in the blunt spatula group variable amounts of CK12 staining was noted in the cornea (Fig. 2A). DAPI staining of wholemounts verified no erosion on corneal surface. Positive CK8 staining was observed in Algerbrush and thermal injury groups at three months, while spatula scraped corneas showed less CK8 staining and unwounded corneas devoid of any (Fig. 2A). Cytokeratin 12/8 immunostaining of frozen sections (four eyes in each group) also revealed the same results (Fig. 2A, data for CK8 not shown).

The ratio of CK12 to CK8 densities for each eye was calculated and the averages were reported for each group. The mean ratio of CK12 to CK8 staining was found to be significantly less in Algerbrush and heat treated corneas compared to unwounded eyes ($P < 0.0001$, Fig. 2B) and spatula group ($P < 0.0001$, Fig. 2B). There was no significant difference in the ratios between Alger alone and Alger plus heat methods ($P = 0.1507$).

To additionally confirm LSCD, immunostaining for MUC5AC (goblet cell-specific mucin) (Pajoohesh-Ganji et al., 2012) was performed on four wholemount samples from each group at two months (mouse anti-MUC5AC, Thermo Scientific, Fremont, CA, 1:200, M.O.M. kit, Vector Laboratories, Burlingame, CA). No goblet cells were observed in unwounded corneas, while other three groups showed variable amounts of mucin staining, confirming the presence of goblet cells and LSCD (Fig. 2A).

Corneal opacity was scored on slit lamp images taken three