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Abstract

The New Zealand White rabbit has been widely used as a model of limbal stem cell deficiency (LSCD). Current techniques for experimental induction of LSCD utilize caustic chemicals, or organic solvents applied in conjunction with a surgical limbectomy. While generally successful in depleting epithelial progenitors, the depth and severity of injury is difficult to control using chemical-based methods. Moreover, the anterior chamber can be easily perforated while surgically excising the corneal limbus. In the interest of creating a safer and more defined LSCD model, we have therefore evaluated a mechanical debridement technique based upon use of the AlgerBrush II rotating burr. An initial comparison of debridement techniques was conducted in situ using 24 eyes in freshly acquired New Zealand White rabbit cadavers. Techniques for comparison (4 eyes each) included: (1) non-wounded control, (2) surgical limbectomy followed by treatment with 100% (v/v) n-heptanol to remove the corneal epithelium (1-2 minutes), (3) treatment of both limbus and cornea with n-heptanol alone, (4) treatment of both limbus and cornea with 20% (v/v) ethanol (2-3 minutes), (5) a 2.5-mm rounded burr applied to both the limbus and cornea, and (6) a 1-mm pointed burr applied to the limbus, followed by the 2.5-mm rounded burr applied to the cornea. All corneas were excised and processed for histology immediately following debridement. A panel of four assessors subsequently scored the degree of epithelial debridement within the cornea and limbus using masked slides. The 2.5-mm burr most consistently removed the corneal and limbal epithelia. Islands of limbal epithelial cells were occasionally retained following surgical limbectomy/heptanol treatment, or use of the 1-mm burr. Limbal epithelial cells were consistently retained following treatment with either ethanol or n-heptanol alone, with ethanol being the least effective treatment overall. The 2.5-mm burr method was subsequently evaluated in the right eye of 3 live rabbits by weekly clinical assessments (photography and slit lamp examination) for up to 5 weeks, followed by histological analyses (hematoxylin &
eosin stain, periodic acid-Schiff stain and immunohistochemistry for keratin 3 and 13). All 3 eyes that had been completely debrided using the 2.5-mm burr displayed symptoms of ocular surface failure as defined by retention of a prominent epithelial defect (~40% of corneal surface at 5 weeks), corneal neovascularization (2 to 3 quadrants), reduced corneal transparency and conjunctivalization of the corneal surface (demonstrated by the presence of goblet cells and/or staining for keratin 13). In conclusion, our findings indicate that the AlgerBrush II rotating burr is an effective method for the establishment of ocular surface failure in New Zealand White rabbits. In particular, we recommend use of the 2.5-mm rotating burr for improved efficiency of epithelial debridement and safety compared to surgical limbectomy.

Keywords
Cornea; wound healing; animal models; New Zealand White rabbit; debridement; rotating burr; ocular surface failure; limbal stem cell deficiency.
1. Introduction

Ocular surface failure arises from a deficiency of the epithelial progenitor cells required to maintain the corneal epithelium. In humans, these epithelial progenitor cells are concentrated within epithelial crypts residing within a narrow transitional zone of tissue known as the corneal limbus, where the cornea adjoins the sclera (Schermer et al., 1986; Shanmuganathan et al., 2007). The term “limbal stem cell deficiency” or its abbreviation, LSCD, is therefore used to describe ocular surface failure (Tseng, 1989). LSCD is characterized by loss of corneal epithelial cells, translocation of conjunctival epithelial cells onto the anterior surface of the cornea and vascularization of the corneal stroma.

Animal models have been vital to the development of therapies for the treatment of LSCD and other corneal disorders (reviewed by (Stepp et al., 2014)). In fact, the current model of corneal epitheliogenesis is founded upon key concepts established in the New Zealand White rabbit (Thoft and Friend, 1983). Subsequently, this species has been used to examine the efficacy of epithelial tissue transplants for the treatment of LSCD (Schwab, 1999). Refinements of this technology over the last two decades have led to the establishment of methods for the treatment of LSCD in several clinics around the world (Harkin et al., 2013; Pellegrini et al., 1997; Sangwan et al., 2012; Schwab, 1999; Shimazaki et al., 2002; Shortt et al., 2007; Zakaria et al., 2010). While there are significant variations in methodology, each treatment strategy is ultimately built around the engraftment of epithelial progenitor cells to replace those lost through disease and trauma. When cultivated *ex vivo* prior to implantation, the epithelial progenitor cells are usually applied in conjunction with donor human amniotic membrane and/or fibrin glue (reviewed by (Shortt et al., 2007)).

The outcomes and limitations of corneal epithelial progenitor cell therapies has encouraged the exploration of alternative progenitor cells including those derived from oral
mucosa (Inatomi et al., 2006), bone marrow, adipose tissue, dental tissue and placental tissue (reviewed by (Harkin et al., 2015)), or corneal/limbal stroma (reviewed by (Funderburgh et al., 2016). In addition, a range of biomaterials have been proposed as alternative epithelial tissue scaffolds to amniotic membrane and fibrin glue (reviewed by (Chirila et al., 2010)). Nevertheless, each new combination of progenitor cells and biomaterials requires testing for safety and efficacy in a preclinical model prior to use in humans. This renewed interest in preclinical studies has led us to reflect on current methods for induction of LSCD in the New Zealand White rabbit.

Our review of the literature reveals occasional treatment with caustic chemicals such as sodium hydroxide (Luengo Gimeno et al., 2007), but more commonly in rabbits, a surgical limbectomy is performed and often in conjunction with application of n-heptanol (Avila et al., 2001; Hirst et al., 1981; Liu et al., 2013; Reinshagen et al., 2011; Schwab, 1999; Shapiro et al., 1981; Ti et al., 2002) or other organic solvents including methanol (Brown et al., 2014), to remove the corneal epithelium. While sodium hydroxide creates chemical burns similar to those associated with domestic and industrial accidents, the depth of injury and subsequent pathology is difficult to control thus increasing the potential for variable results. Likewise, when performing a limbectomy, considerable care must be taken to avoid perforating the anterior chamber of the eye. Either scenario can potentially lead to a greater number of animals being required which is deleterious from both an economic and ethics perspective. As an alternative strategy, we have therefore examined a mechanical debridement technique based upon application of the AlgerBrush II rotating burr.

The AlgerBrush II rotating burr tool (The Alger Company Inc, Texas, USA) was originally developed for the purpose of removing rust-rings arising from metallic foreign bodies lodged in the cornea, but is also promoted by the manufacturer for use in other corneal surgeries including removal of pterygia. The broadening of clinical applications has led to
availability of a variety of burr shapes, sizes and coatings including a 1-mm tungsten burr tip and a rounded 2.5-mm diamond encrusted burr tip (Figure 1). Experimentally, these tools have been widely used to generate partial epithelial defects (reviewed by (Stepp et al., 2014)). A more limited number of studies have utilized the AlgerBrush II to induce LSCD in mice (Afsharkhamseh et al., 2015; Ksander et al., 2014; Meyer-Blazejewska et al., 2011), but the suitability of this technique for other species including rabbits is unclear. We therefore presently evaluate the suitability of the AlgerBrush II as a tool for inducing LSCD in the New Zealand White rabbit. Two different strategies based upon use of the Algerbrush II are evaluated. Either a 2.5-mm rounded burr or a 1-mm pointed burr is applied across the entire limbus, followed by use of the rounded burr to remove the corneal epithelium. These techniques are initially compared using freshly acquired cadaveric tissue, with the optimal method subsequently being validated in a cohort of live rabbits. Moreover, the initial study in cadaveric tissue is made in comparison with the current standard technique (n-heptanol applied in conjunction with a limbectomy) and variations based upon this method (treatment with either n-heptanol or ethanol alone).

2. Materials and methods

2.1. Ethics statement

All procedures were conducted in accordance with the ‘Animal Care and Protection Act’ (Queensland State Government, Australia, 2001), ‘Australian Code for the Care and Use of Animals for Scientific Purposes’ (8th Edition, 2013) and the ‘ARVO Statement for Use of Animals in Ophthalmic and Vision Research’.
2.2. Sourcing and handling of rabbit cadavers

Rabbit cadavers (female New Zealand White rabbits weighing between 2.5-3.0 kg) were sourced within 30 minutes following their use by non-ophthalmic surgical training workshops conducted within the same research facility. All procedures were conducted within 1-hour post-mortem.

2.3. Comparison of debridement techniques

A total of 20 cadaveric rabbit eyes were experimented on while still in situ. An additional 4 eyes were immediately enucleated and fixed in 10% (v/v) neutral buffered formalin (3.7% w/v formaldehyde) for subsequent use as histological controls. A different debridement technique was used for the left and right eye within each pair in order to reduce the potential for animal specific responses. An experienced ophthalmic surgeon (F.J.L) performed all the procedures with the aid of a speculum and surgical microscope. Each debridement was preceded by a 360° conjunctival peritomy, approximately 1.5-mm beyond the limbus, with dissection towards the limbus. One of the following five procedures was then performed on each eye (n = 4 for each group): (1) a 360° limpectomy followed by treatment of the cornea with a cotton-bud soaked in 100% (v/v) n-heptanol (1-2 minutes; Sigma Cat. No. 72954), (2) treatment of the cornea and limbus with n-heptanol, (3) treatment of the cornea and limbus with a cotton-bud soaked in 20% (v/v) ethanol (2-3 minutes), (4) a 360° superficial limbal keratectomy using an AlgerBrush II fitted with a 1-mm tungsten burr (corneal rust removal burr, Rumex International Cat. No. 16-140), followed by removal of the remaining corneal epithelium using a 2.5-mm round-ended, diamond-dusted burr (Rumex International Cat. No. 16-051-2.5B), or (5) a 360° superficial limbal keratectomy followed by complete epithelial debridement using only the 2.5-mm round-ended burr. A visual comparison of the two types of burr tip during application to the limbus is provided in Figure 1. Following all treatments,
the ocular surface was rinsed with saline and any noticeable debris removed using a lightly moistened cotton-bud. Further specific details for each surgical procedure are as follows.

Prior to performing the 360° limbectomy, a trephine blade was used to create a superficial radial incision approximately 1.5 mm within the limbal boundary. Starting from the 12 o’clock position, a guarded blade was then used to create a partial-thickness circular incision within the peripheral cornea. A crescent blade was subsequently used to perform a superficial lamella dissection toward the limbus. Scissors were used to complete the excision.

During application of solvents (n-heptanol or ethanol), the soaked cotton-bud was applied to the ocular surface with light pressure in a circular motion until the tissue was rendered slightly opaque. The time taken to achieve this state was slightly longer when using ethanol (2-3 minutes compared with 1-2 minutes for n-heptanol). The eye was then immediately rinsed with saline three times to remove excess solvent. A corneal epithelium spatula was then used to scrape away tissue that became dislodged during treatment with solvents.

During mechanical debridement of the limbus, each burr tip was applied with light pressure in a circular motion starting at the 12 o’clock position and slowly moving around the entire periphery of the cornea until a region spanning approximately 1.5 mm beyond and 1.5 mm within the limbal boundary had been treated.

Immediately following debridement, all eyes were enucleated and placed in neutral buffered formalin (3.7% formaldehyde). The anterior chamber was perforated via the sclera using a syringe needle to assist penetration of the fixative.

2.4. Histological techniques

Enucleated rabbit eyes were routinely fixed for 2-3 hours (3.7% formaldehyde) before being processed through graded alcohols, xylene and into paraffin. The anterior segment of each eye
was removed and sectioned into two to four strips of approximately equal width (3 to 4-mm) before embedding into paraffin blocks. Approximately a dozen paraffin sections measuring approximately 3 µm in thickness were cut from each block and mounted on treated glass microscope slides (Superfrost™ Ultra Plus Adhesion, Thermo Scientific).

The initial comparison of debridement techniques was subsequently made using 3 hematoxylin & eosin (H&E) stained sections from each block, with each section containing a minimum of 2 pieces of tissue spanning the limbus and central cornea.

Tissue samples obtained from live wounded animals were fixed, processed, sectioned and stained as above, but with an equal number of sections being stained using the periodic acid-Schiff (PAS) technique to investigate the potential presence of goblet cells. Remaining sections were subsequently used to confirm epithelial phenotype by immunostaining for keratin 3 (a differentiation marker for corneal epithelial cells) and keratin 13 (a differentiation marker for conjunctival epithelial cells). Epitope retrieval (ER) was performed prior to immunostaining by immersing deparaffinized slides in CINtec® Histology Kit (Roche, Cat. No. 9511) epitope retrieval solution (1-mm EDTA/10 mM Tris buffer, pH 9.0) for 10 minutes at 85 ºC. The Coplin jar containing slides in ER solution was then placed for a further 20 minutes at room temperature during which time the temperature dropped to approximately 55 ºC. After rinsing in staining buffer (10 mM Tris buffered saline with 0.025% Triton X-100) the slides were transferred to a staining rack placed within a humidified container. Endogenous peroxidases were inactivated by treatment with 0.3% hydrogen peroxide for 10 minutes. After further rinsing in buffer the slides were incubated for 1-hour at room temperature in buffer containing primary antibodies to either keratin 3 (a 1:300 dilution of mouse monoclonal AE5 obtained from Millipore Pty Ltd, Cat. No. CBL218) or keratin 13 (a 1:300 dilution of mouse monoclonal AE8, Abcam Pty Ltd, Cat. No. ab16112). Binding of primary antibodies was subsequently detected using a horseradish peroxidase/polymer-
conjugated goat-anti-mouse detection system (a component of CINtec® Histology Kit, Roche, Cat. No. 9511). Negative controls were performed by excluding the primary antibody incubation step. Positive controls consisted of non-wounded tissue sections stained with antibodies to either keratin 3 or keratin 13. The chromogen used was diaminobenzidine (DAB). Nuclear counterstaining was performed by treatment for 5 minutes with Gill’s hematoxylin solution (United Biosciences, Carindale, Queensland, Cat. No. G1-1L), followed by rinsing in Scott’s tap water substitute (United Biosciences, Carindale, Queensland, Cat. No. SCOT-1L). After dehydration through graded alcohols and clearing in xylene, the slides were mounted in plastic mounting medium and imaged using an Olympus BX41 microscope equipped with a 20x/0.8 NA UPlanApo oil-immersion lens and Nikon Ri1 digital camera. Images were acquired using NIS Elements version 4 and all post-acquisition image modifications was undertaken using Adobe Photoshop CS5 (Version 12.0, Adobe Systems Inc.). Image modifications consisted of (in order) initial re-sizing (to 8 x 6 cm at 300 dpi), cropping, montage creation, threshold optimization using levels function, and labeling.

2.5. Scoring of histological sections obtained from cadaveric tissue

The entire collection of H&E stained slides obtained from the 24 cadaveric rabbits (72 slides in total, consisting of 12 slides and at least 24 sections per treatment cohort, representing at least 6 H&E sections per eye) were de-identified and coded by placing opaque stickers with random numbering (1-72) above the original slide labels. The identity of each slide was then masked before being presented in random order to a panel of four independent observers (D.G.H, E.N., F.J.L and J.W). The degree of epithelial tissue displayed on each slide was rated using a 5-point scoring system (i.e. values between 0 to 4, according to criteria described in Table 1). A separate score was assigned for the cornea and limbus. After decoding the data by removal of stickers, the mean score for each wounded eye (represented
as individual dots in Figure 2) was then calculated as the average of values provided by the four independent observers (n = 4). The mean value for each treatment cohort (represented as lines in Figure 2) was calculated as the average value obtained for each set of four eyes. The data was analyzed for statistical significance using a Kruskal-Wallis test followed by Dunn’s multiple comparisons test (a non-parametric test based upon ranking of values). The statistical analysis/graphing software used was Prism 6 (GraphPad Software).

Table 1. Scoring system used for assessment of epithelial structure following experimental debridement of cadaveric rabbit corneas *in situ*.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Stratified epithelium with little or no evidence of cell loss.</td>
</tr>
<tr>
<td>3</td>
<td>Partially stratified epithelium. Clear evidence of epithelial cell loss.</td>
</tr>
<tr>
<td>2</td>
<td>Partial coverage with epithelial monolayer.</td>
</tr>
<tr>
<td>1</td>
<td>Evidence of non-nucleated cellular material (nucleated cells potentially present above or below plane of sectioning).</td>
</tr>
<tr>
<td>0</td>
<td>Absence of cellular material.</td>
</tr>
</tbody>
</table>

A separate score was assigned to the cornea and limbus for each H&E stained slide (minimum of 2 sections per slide from different levels within the paraffin block). Three masked slides were examined per eye. Four assessors were employed. An average score was estimated in instances where results were considered to be variable across the region of interest (e.g. such as in the case of an incomplete limbectomy where a portion of limbal epithelium was retained).

2.6. Routine care of live rabbits

Live animal studies were conducted using 4 male New Zealand White rabbits weighing between 2.5 to 3.0 kg. All rabbits were sourced from a commercial laboratory breeding facility. Routine health checks were performed on arrival and a radio-frequency identification microchip (MyChip, Provet Pty Ltd Australia) was implanted subcutaneously into the scruff of the neck. Rabbits are designated as environmental pests in the Australian state of Queensland and so therefore were housed in a Queensland Government Declared Pest Permit Approved Premise (Permit no.0526-01-SRC-003). The rabbits were housed in individual
rabbit cages (Tecniplast Australia Pty Ltd, Australia) and supplied with straw bedding, shredded paper and environmental enrichment (cardboard boxes and plastic toys). The food supply consisted of a commercial, laboratory-grade, high-fiber, and low-starch, pelleted rabbit diet (Specialty Feeds, Western Australia) supplemented with fresh fruit and vegetables. Food and water were supplied *ad libitum* and levels checked daily.

### 2.7. Anesthesia

Rabbits were pre-medicated with 50 µg/kg buprenorphine (Temgesic® 300µg/mL, Jurox Pty Ltd, Australia) subcutaneously, approximately 20 minutes prior to general anesthesia. Anesthetic induction was performed using an injectable combination of 15 mL/kg ketamine (Ilium Ketamil® 100mg/mL, Troy Laboratories Australia Pty Ltd) and 0.25 mg/kg medetomidine (Domitor® 1 mg/mL, Pfizer Animal Health, NSW Australia). A 24G intravenous cannula (Optiva® 24G IV Catheter Radiopaque, Medex Medical Ltd, Great Britain) was introduced into the marginal ear vein to allow for intravenous surgical maintenance fluid therapy. General anesthesia was maintained via a size 1 mask (Vetquip, Castle Hill, NSW Australia) under 1-2% isoflurane (Attane, Bayer Australia) through an Isoflurane Tec 3 vaporizer fitted to a MQV1100 Anesthetic Machine (Mediquip Pty Ltd, Australia).

### 2.8. Surgical procedures

Debridement of the entire limbal and corneal epithelium using the 2.5-mm rounded burr tip was essentially performed as reported for cadaveric tissue with the following additions. All surgical instruments and swabs were either purchased sterile or sterilized prior to surgery by autoclave. The handle of the AlgerBrush II debridement tool was decontaminated by spraying with 70% ethanol. An area measuring approximately 50-100 cm² around each eye was
decontaminated using a sterile surgical swab doused in 10% w/v povidone-iodine (Betadine® Antiseptic Solution, Mundipharma B.V., Netherlands). A sterile field was created using a nylon surgical drape containing a circular hole measuring approximately 25 cm². Each debridement procedure including the initial peritomy took approximately 40 minutes to complete (determined visually). This time can be nearly halved (to approximately 25 minutes) if the rabbit’s eye is proptosed prior to surgery. Following epithelial debridement, the nictitating membrane was secured to the lower temporal side eyelid using a 4.0 nylon suture.

2.9. Postoperative care

During recovery from anesthesia each animal was supplied with oxygen and fitted with a 10 cm diameter soft cat recovery Elizabethan collar to reduce further trauma to the injured eye by incidental cleaning or brushing against objects. All animals were awake within 1-hour post-surgery and responsive to food and water within 2-3 hours. Post-operative pain management was performed using a multi-modal analgesic protocol. This consisted of alternating morning and afternoon subcutaneous injections of 0.05 mg/kg meloxicam (Metacam® 5 mg/mL, Boehringer Ingelheim Vetmedica, Inc.) and 50 µg/kg buprenorphine (Temgesic® 300 µg/mL, Jurox Pty Ltd, Australia) until the morning of the 5th day. In addition, a combination eye ointment preparation consisting of 5 mg/g neomycin sulfate, 5000 IU/g polymixin B sulfate, 2.5 mg/g prednisolone and 50 mg/g sulfacetamide sodium (Amacin® Eye and Ear Ointment, Jurox Pty Ltd, Australia) was supplied twice daily throughout the entire post-operative period, as well as after each clinical examination. The suture securing the nictitating membrane to the lower temporal eyelid was removed after 7 days. Upon completion of studies, each animal was euthanized by slow intravenous injection with 325 mg/kg of sodium pentobarbital.
2.10. Clinical assessments

Clinical assessments were performed weekly for up to 5 weeks. Two to three drops of 0.5% proparacaine (Alcaine® 0.5% eye drops, Alcon Laboratories Pty Ltd Australia) were inserted into each eye approximately 5 minutes prior to each assessment. A speculum was inserted to provide a clearer view of the corneal margins and a sterile cotton-bud used to retract the nictitating membrane temporarily if required. Each examination commenced by taking a photograph to record the presentation of the ocular surface. A Canon EOS 6D digital SLR camera equipped with a Canon macro lens (EF 100 mm 1:2.8 L IS USM) and Canon Macro Ring Lite MR-14EX II flash was used (Camera settings: ISO 400, 1/100, f13). A slit lamp examination was subsequently performed to assess changes in corneal structure including stromal edema and epithelial integrity using a Keeler Classic portable slit lamp. A fluorescein paper strip soaked in saline was inserted beneath the upper eyelid and held with light pressure from a gloved hand for approximately 1 minute prior to examination under the slit lamp’s cobalt lamp. A yellow lens filter and blue flash filter were applied during photography (Camera setting: ISO 1600, 1/60, f8.0) under cobalt lamp illumination.

2.11. Analysis of clinical photographs

The approximate size of epithelial defects in each wounded eye at a given time point was determined using ImageJ (Version 1.48v; National Institutes of Health, USA) image analysis software. Briefly, the relative size of each defect was measured by tracing images of fluorescein-stained eyes with a computer mouse (using the freehand measure function) and then expressing these values as a percentage of the total corneal area (as defined by an ellipse outlining the approximate corneal margin). The time course of changes in percentage defect for each animal was plotted using Prism 6 (GraphPad).
3. Results

3.1. Comparison of debridement techniques

The combined histological scores obtained from four blinded observers revealed significant differences in efficiency of epithelial debridement between techniques tested when trialed using cadaveric tissue (Figure 2). Application of 100% n-heptanol consistently removed evidence of viable corneal epithelial cells (judged by complete absence of cell nuclei) in the total of 8 eyes examined (Figure 2A). This method, however, proved to be far less efficient at removing the limbal epithelium with all 4 eyes examined displaying only minimal signs of damage (Figure 2B). By comparison, treatment with 20% ethanol (a technique used clinically prior to photorefractive surgery) (Shah et al., 2001) was even less efficient than n-heptanol at removing epithelial cells from either the cornea or limbus. No attempt was made to test ethanol at a higher concentration since this alcohol permeates tissue more rapidly than n-heptanol and in fact is histochemically a faster method of tissue fixation than formalin.

Performing a surgical limpectomy obviously has greater potential for removing epithelial tissue, but requires considerably more care than either of the solvent-based techniques. As such, the anterior chamber was perforated on at least one occasion during this study using cadaveric tissue in situ. Unexpectedly, however, on two occasions, significant traces of nucleated cells remained within the limbus following limpectomy. An example of this outcome is displayed in Figure 3B (within limbus). Likewise, nucleated epithelial cells were retained within the peripheral cornea of one eye following application of the 1-mm burr tool (Figure 3E, within peripheral cornea). In contrast, least evidence of epithelial tissue was consistently retained within either the corneal or limbal compartments following application of the 2.5-mm rounded burr tool. This efficiency (compared to non-wounded controls) was confirmed as statistically greater than for all other techniques tested both within the central
cornea as well as the limbus (p < 0.01). The depth of corneal wound created by the 2.5-mm burr extended to include portions of basement membrane (as revealed in H&E and PAS stained sections by light microscopy), but little or no tissue loss was evident within the adjacent corneal stroma. Within the limbus, however, the depth of wound extended a further 50-100 µm to include the superficial layer of loose connective tissue including the majority of blood vessels servicing the limbal stem cell niche (compare right side panels of Figure 3 A and F).

3.2. Validation of wound model in vivo

Encouraged by the data obtained using cadaveric tissue, we proceeded to examine the progression of wounds achieved over 5 weeks after applying the 2.5-mm burr tool alone across the entire ocular surface of one eye each in three live rabbits (hereby referred to as R1, R2 and R3). For comparison, in one additional rabbit the corneal epithelium was removed as previously, but the limbus left in intact (R4). Subsequent analysis of fluorescein-stained images using ImageJ revealed a consistent pattern of slow re-epithelialization in all three completely debrided eyes, with each retaining a defect of approximately 40% by 5 weeks (Figure 4). In contrast, the single eye with an intact limbus had nearly completely re-epithelialized within 2 weeks (Figure 4 and Figure 5). The slower rate of re-epithelialization observed with full wounds was accompanied by a sustained level of corneal haze and vascularization (Table 2). The degree of vascularization (beginning around 2-3 weeks) varied between 2 to 3 quadrants with the majority of vessels appearing to be quite superficial (Figure 5).
Table 2. Summary of clinical observations and histological data.

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Wound</th>
<th>Epithelial defect</th>
<th>Corneal haze</th>
<th>Neovascularization (Number of quadrants)</th>
<th>Conjunctivalization</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>Complete debridement of both corneal and limbal epithelia using the 2.5 mm burr</td>
<td>39.8% at 5 weeks</td>
<td>Yes</td>
<td>2</td>
<td>Yes PAS(-)/K13(+) K3 (a few cells)</td>
</tr>
<tr>
<td>R2</td>
<td>As above</td>
<td>41.6% at 5 weeks</td>
<td>Yes</td>
<td>3</td>
<td>Yes PAS(+)/K13(+) K3 (a few cells)</td>
</tr>
<tr>
<td>R3</td>
<td>As above</td>
<td>45.5% at 5 weeks</td>
<td>Yes</td>
<td>3</td>
<td>Yes PAS(+)/K13(+) K3 (a few cells)</td>
</tr>
<tr>
<td>R4</td>
<td>Incomplete (2.5 mm burr applied to cornea only)</td>
<td>3.5% at 2 weeks</td>
<td>No</td>
<td>0</td>
<td>No PAS(-)/K13(-) K3 (+)</td>
</tr>
</tbody>
</table>


Histology revealed significant differences in the ocular surface of all four wounded rabbits (R1 to R4) compared to non-wounded control tissue (Figure 6). H&E staining revealed partial coverage of the cornea with varying levels of stratified squamous epithelial cells. Structures resembling goblet cells (a marker of conjunctival epithelial tissue) were observed in R2 and R3, but not R1 or R4. Subsequent staining for neutral mucins using the periodic acid-Schiff (PAS) method confirmed the presence of goblet cells in only R2 and R3. Numerous inflammatory cells were evident within the corneal stroma of rabbits R1, R2 and...
R3, but not R4. The stroma of R2 and R3 were noticeably more vascularized than that of R1, and the stroma of R4 appeared avascular. Further analysis by immunohistochemistry (Figure 7) revealed the presence of a few keratin 3-positive cells within the healing epithelia of R1, R2 and R3. All three epithelia, however, consisted of predominantly keratin 13-positive cells, with darker staining being observed in R2 and R3 compared to that found in R1. In contrast, the healing epithelium of R4 consisted entirely of keratin 3-positive cells with no traces of keratin 13 being detected.

4. Discussion

Animal models of LSCD rely upon the efficient removal of corneal epithelial progenitor cells. Historically, this condition has been achieved by performing a limbectomy and/or treatment chemicals including sodium hydroxide (Luengo Gimeno et al., 2007), n-heptanol (Schwab, 1999) or methanol (Brown et al., 2014). While these techniques have generally been used to good effect, it is technically challenging to achieve an adequate and reproducible level of tissue damage. Some animals may therefore need to be excluded from studies owing to accidental perforation of the anterior chamber or spontaneous healing due to inadequate damage. In either case, additional animals are required thus significantly increasing the time and cost associated with the project. Moreover, the inefficient use of animals is undesirable from an animal research ethics perspective. Thus prior to embarking on our own experimental treatments for LSCD in the New Zealand White rabbit, we have evaluated an alternative technique based upon application of the AlgerBrush II rotating burr. Our results demonstrate that the AlgerBrush II is a more efficient method for removing the limbal epithelium than performing a limbectomy or treatment with n-heptanol (Figure 2). In particular, application of
the 2.5-mm rounded burr tool was found to remove the corneal and limbal epithelia most effectively (Figures 2 and 3) from fresh cadaveric tissue and routinely led to classic symptoms of LSCD (corneal vascularization and conjunctivalization) when tested in a limited number of live animals (Figures 4, 5 and 6).

While the AlgerBrush II rotating burr has been widely used as a tool for creating partial wounds in the corneal epithelium of animals (Stepp et al., 2014), surprisingly few studies have attempted to induce LSCD using this device. The exceptions are a limited number of studies conducted in mice (Afsharkhamseh et al., 2015; Ksander et al., 2014; Meyer-Blazejewska et al., 2011). In the first of these studies (Meyer-Blazejewska et al., 2011), a 0.5-mm rotating burr was applied to the entire limbal and corneal epithelium. The development of LSCD was subsequently confirmed by demonstration of goblet cells in PAS stained sections of corneal tissue. The second study in mice (Ksander et al., 2014), also used the 0.5-mm burr and reported similar results by demonstrating loss of the corneal epithelial cell marker keratin 12. Presumably both groups had good reasons for choosing a rotating burr over more conventional methods but a comparison of techniques was not presented. A brief communication (Afsharkhamseh et al., 2015), however, has recently appeared in the literature comparing use of the 0.5-mm rotating burr in mice to a mechanical method based upon scraping with a blunt spatula. The effects of the rotating burr were examined alone and in conjunction with a controlled thermal injury delivered to the limbus using an ophthalmic cautery device. Subsequent analysis of excised whole-mounted corneas by immunohistochemistry demonstrated that the rotating burr method resulted in a significantly greater reduction in keratin 12 than that achieved by scraping with a spatula. The presence of conjunctival tissue on the corneal surface in this previous study was confirmed by immunostaining for keratin 8 and MUC5AC. Interestingly, use of the rotating burr tool alone (without cauterization) was sufficient to generate optimal results.
The results of the present study in rabbits are consistent with those achieved in mice in demonstrating the effectiveness of the AlgerBrush II rotating burr for removing the corneal and limbal epithelium. In addition, our studies in cadaveric tissue illustrate some of the variable outcomes that can be produced using more conventional methods. In particular, significant traces of intact limbal epithelium are occasionally observed following a limbectomy. While these eyes were not followed for evidence of recovery in a living animal, the retention of limbal tissue would be expected to increase the likelihood of spontaneous healing. It is possible that with more experience we may have been able to increase the efficiency of the limbectomy procedure, however, performing a limbectomy still requires considerably more care than that required during application of the rotating burr.

During initial selection of a rotating burr, we speculated in a similar manner to prior researchers (Afsharkhamseh et al., 2015), that the undulating crypt-like structure of the limbal stem cell niche may hamper complete removal of more basal progenitor cells. We therefore hypothesized that the finer 1-mm pointed burr tool would penetrate more deeply, thus leading to more efficient removal of the more basal epithelial cells. In practice, however, there was very little difference between the efficiency of the pointed 1-mm burr and the 2.5-mm rounded burr. Indeed on one occasion, use of the finer tipped burr resulted in a small area of peripheral corneal epithelium being retained close to the limbus (Figure 3E). Upon reflection, we consider that this minor error most likely occurred as a result of losing track of which areas had been wounded when switching between burr tools. Moreover, use of the finer burr tool is inherently more likely to leave small non-wounded gaps than the 2.5-mm rounded tool. Our current experimental protocol in live animals has therefore been modified to include a fluorescein staining step (viewed under cobalt lamp illumination) to check for small traces of intact epithelium following debridement. Live confocal imaging or optical coherence tomography (OCT) might also be used as tools for evaluating wound depth intra-operatively.
Ultimately, adoption of the rotating burr method for induction of LSCD will be based upon the consistency of results obtained. To this end, we have demonstrated a similar slow rate of re-epithelialization over 5 weeks in three consecutively wounded animals (Figure 4). Likewise, all three animals developed a conjunctivalized corneal surface based upon positive immunostaining for keratin 13 and poor staining for keratin 3 (Figure 7). Nevertheless, goblet cells were only present in two wounded corneas (Figure 6) and the cornea without goblet cells (R1) displayed noticeably less vascularization (Figure 5). In fact, the histological appearance of the epithelium generated in R1 is reminiscent of the pseudo-transdifferentiation of conjunctival tissue observed when there is incomplete removal of basal limbal epithelial cells (Kruse et al., 1990). Given the consistent scarcity of intact epithelium following application of the AlgerBrush II to cadaveric tissue (Figure 3), the apparent retention of corneal-limbal epithelial cells on the ocular surface of live animals is most likely related to some dislodged limbal epithelial progenitor cells reattaching, owing to inadequate rinsing. In other words, it may well be possible to mimic the effects of seeding of small epithelial clusters akin to the recent innovation of seeding small fragments of autologous limbus for the treatment of LSCD (i.e. simple limbal epithelial transplant or SLET (Sangwan et al., 2012)). Whatever the explanation, since traces of keratin 3 positive cells were sporadically observed in all completely wounded animals (R1 to R3), we must conclude that the level of epithelial cell removal achieved using the Algerbrush II is less than 100%. This conclusion is consistent with prior data produced in mice (Afsharkhameh et al., 2015).

A final issue for consideration by researchers is the time required to conduct an efficient debridement. The greatest number of rabbits that we have wounded in a single day using the Algerbrush technique is 3, even when the eye is proptosed. Nevertheless, our preclinical studies of new therapies (in progress) involve a range of ancillary procedures including regular blood collections and clinical photography. Depending upon the study
design, a competent research surgeon supported by a team of 2-3 research staff might well be able to complete debridements on 4-6 rabbits per day (allowing for induction and post-operative care). We prefer to limit ourselves to no more than 3 surgeries per day for logistical reasons (e.g. frequency of post-operative blood sampling) as well as the general welfare of the rabbits during the post-operative recovery period (i.e. more personalized care).

5. Conclusions

The Algerbrush II rotating burr tool is at least as effective as n-heptanol for removing the rabbit corneal epithelium, and offers a safer and more effective method for removing the limbal epithelium. For best results, however, it is recommended that the degree of epithelial debridement be checked by fluorescein stain, and that the ocular surface is rinsed well following debridement to reduce the likelihood of dislodged cells reseeding onto the ocular surface. With attention to these details, we propose that the Algerbrush II rotating burr will become a standard tool for induction of LSCD in the New Zealand White rabbit.

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7. References


8. Figure legends

**Figure 1.** Demonstration of the different AlgerBrush II rotating burr tools as used during application to the corneal limbus of cadaveric rabbit eyes. (A) 1.5-mm pointed burr. (B) 2.5-mm rounded burr. The first strategy employed the 1.5-mm burr tool to debride the limbus, followed by application of the 2.5-mm burr tool to debride the corneal epithelium. The second strategy employed the 2.5-mm burr tool alone to debride the entire epithelium (cornea and limbus).

**Figure 2.** Comparison of debridement techniques as demonstrated by histological score. Dot plots represent the individual mean values obtained for each wounded eye (with 6 H&E stained sections being scored per eye). Bars represent the mean value overall for each wound method (n=4). The degree of significant difference to control (p < 0.05 or p < 0.01) was determined using a Kruskal-Wallis test followed by Dunn’s multiple comparison’s test (a non-parametric test based on ranking).

**Figure 3.** Examples of histology observed following each wounding technique. Left panels display an H&E stained section of central cornea. Right panels display corresponding appearance of limbus, or peripheral cornea (only in the case of part E) in same tissue sections. Top to bottom displays typical appearance of (A) control tissue fixed without wounding, (B) results achieved following limbectomy and debridement of cornea using 100% n-heptanol (on this occasion a small amount of limbal epithelium was retained), (C) debridement of cornea and limbus using 100% n-heptanol alone, (D) debridement of cornea and limbus using 20% ethanol, (E) debridement using a combination of 1.5-mm pointed (for limbus) and 2.5-mm
rounded (for cornea) rotating burr tools (right image is of peripheral cornea), (F) complete debridement of cornea and limbus using the 2.5-mm burr tool.

**Figure 4.** Time course of wound progression following epithelial debridement using the 2.5-mm burr tool (epithelial defect as quantified from fluorescein stained images using ImageJ). Five weeks after complete debridement of the corneal epithelium (including limbus), all three animals retained an epithelial defect of approximately 40% (animals R1, R2 and R3). In sharp contrast, only a minor defect (3.5%) remained two weeks after debridement of the corneal epithelium alone (animal R4).

**Figure 5.** Gross appearance of eyes after being wounded using only the 2.5-mm rotating burr. As previously, animals R1 to R3 received full debridement, whereas the limbus was left intact for R4. Images illustrate the appearance of eye at end of study (either 5 weeks for R1 to R3 or 2 weeks for R4). The corresponding panels at right (as viewed under cobalt light following staining with fluorescein) highlight in green the edges of the epithelial defect that remained.

**Figure 6.** Histochemical stains (H&E and PAS stain) demonstrate evidence of a conjunctivalized ocular surface in 2 out of 3 eyes, 5 weeks after receiving Algerbrush (2.5-mm rounded tool) debridement of the entire corneal epithelium (including limbus). **Controls:** (A) H&E stained section of normal central cornea, (B) PAS stained section of normal central cornea, (C) H&E stained section of normal conjunctival tissue, (D) PAS stained section of normal conjunctival tissue. Wounded eyes (R1 to R4) correspond to same eyes as illustrated in Figures 4, 5 & 7. Label “GC” and arrows indicate goblet cells revealed by PAS staining in control conjunctiva (D), but only in two of completely wounded eyes (R2 and R3).
Figure 7. Immunohistochemistry confirms conjunctivalized ocular surfaces in all 3 eyes at 5 weeks after complete epithelial debridement (2.5-mm rounded tool applied to both cornea and limbus) Controls: (A to C) normal central cornea stained for negative control (primary antibody omitted), keratin 3 and keratin 13. (D to F) normal conjunctiva stained for negative control, keratin 3 and keratin 13. Wounded eyes (R1 to R4) correspond to same eyes as illustrated in Figures 4, 5 and 6. While all three completely wounded eyes (R1 to R3) expressed almost exclusively keratin 13, the partially wounded eye (R4) regenerated an epithelium expressing only keratin 3.
**A**

**Cornea**

![Graph showing comparison of different debridement methods on cornea score.](image)

- Control
- Limbectomy + 100% n-Heptanol
- 100% n-Heptanol
- 20% Ethanol
- 1 mm burr + 2.5 mm burr
- 2.5 mm burr

Statistical significance: p < 0.01

**B**

**Limbus**

![Graph showing comparison of different debridement methods on limbus score.](image)

- Control
- Limbectomy + 100% n-Heptanol
- 100% n-Heptanol
- 20% Ethanol
- 1 mm burr + 2.5 mm burr
- 2.5 mm burr

Statistical significance: p < 0.05, p < 0.01
Li et al - Highlights

- The Algerbrush II is an effective tool for induction of LSCD in rabbits.
- Optimal results are achieved using a 2.5-mm rounded burr tool.
- The rabbit cornea becomes conjunctivalized and vascularized within 5 weeks.