NorLeu\textsuperscript{3}A(1–7) Accelerates Clear Corneal Full Thickness Wound Healing

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\textbf{PURPOSE.} We evaluated the effect of the renin angiotensin system (RAS) peptide NorLeu\textsuperscript{3}A Angiotensin (1–7) (NLE) formulated in a viscoelastic gel (USB004) on the healing of full-thickness corneal injuries.

\textbf{METHODS.} Dutch pigmented rabbits received conjunctival administration of 0.3% USB004, 0.03% USB004, or vehicle-control to healthy and full-thickness injured eyes administered once daily for 28 consecutive days. Safety was evaluated using IOP measurement, slit-lamp examination, and confocal microscopy. Evaluations for both efficacy studies included an oblique light examination, modified Seidel test (Seidel test with gentle ocular pressure) as well as during elevated IOP test, confocal microscopy imaging, and histologic analysis.

\textbf{RESULTS.} Application of 0.3% USB004, 0.03% USB004, and vehicle-control was safe in healthy and incised eyes. Further, application of 0.3% and 0.03% USB004 following full-thickness corneal incision resulted in a 2-fold acceleration of resolution of edema and inflammation, reduction in duration of wound leakage on a modified Seidel test (Seidel test with gentle ocular pressure) as well as during elevated IOP test, and healing with near normal architecture without evidence of fibrosis and angiogenesis when compared to vehicle-control animals.

\textbf{CONCLUSIONS.} Topical ocular application of 0.3% and 0.03% USB004 promotes full-thickness corneal wound healing without the evidence of fibrosis and angiogenesis. Further studies are warranted to determine the cornea-specific mechanism of action(s) that promotes regeneration leading to clear corneal healing.

Keywords: renin-angiotensin system, corneal injury, eye, corneal healing, corneal incision, progenitor cells, NorLeu\textsuperscript{3}A(1–7)

Wound healing is categorized into three phases: inflammation, proliferation, and maturation. One major component of wound healing is the sprouting of angiogenic capillaries which invade into a fibrin/fibronectin rich microenvironment.\textsuperscript{1} Unlike dermal tissue reparative processes, corneal wound healing is more complex due to organization and differentiation of the corneal substructures requiring the absence of vasculogenesis.\textsuperscript{1,4} The cornea possesses an orderly arrangement of stromal collagen fibrils and the absence of blood vessels.\textsuperscript{5–7}

Inadequate or incomplete healing of the cornea after injury can result in decreased or lost vision.\textsuperscript{8–10} Corneal wounds arise from surgical procedures (e.g., transplants,\textsuperscript{11,12} incisions for cataract removal and intraocular lens implantation,\textsuperscript{3,14} and laser-assisted in situ keratomileusis\textsuperscript{5,16}), infections (e.g., ulcers\textsuperscript{17–19}), and traumatic injury (e.g., lacerations, perforations\textsuperscript{20,21}). Currently these wounds are repaired surgically using nylon sutures, adhesives,\textsuperscript{22,23} amniotic membrane grafts,\textsuperscript{24,25} or tectonic grafts.\textsuperscript{26,27}

Discovery of the actions of angiotensin 1–7 [A(1–7)], a component of the renin-angiotensin system (RAS), resulted in the identification of a novel axis of RAS involving angiotensin-converting enzyme-2 (ACE2)/A(1–7)/Mas receptor (MasR). This protective axis counteracts the traditional proliferative, fibrotic, proinflammatory, and hypertrophic effects of the ACE/AII/Angiotensin II Type I Receptor (AT1R) axis of RAS.\textsuperscript{3,28} Local RAS components exert regulatory effects on stem cell recruitment and cellular proliferation, thus accelerating tissue regeneration.\textsuperscript{29} Exogenous application of angiotensin peptides has shown accelerated healing of dermal injuries and epidermal repair by regulation of hematoipoiesis and development of epithelial progenitor cells.\textsuperscript{30–36}

An analog of A(1–7), NorLeu\textsuperscript{3}A(1–7) (NLE), was found to be more potent than A(1–7) and Angiotensin II (AII) in accelerating wound repair. NorLeu\textsuperscript{3}A(1–7) is a stimulator of progenitor cells, which differentiate into specialized cells based on tissue requirements.\textsuperscript{35,37} Evaluation in several preclinical models of skin repair demonstrated topical treatment with NLE to be
effective in achieving complete healing of thermal and radiation burns, as well as reducing wound size, wound dehiscence, and scar formation.\(^{37,38}\)

We present results from three studies with NLE formulated in a viscoelastic gel (USB004) to evaluate safety (in normal corneas) and efficacy (in a rabbit corneal wound model). To our knowledge, this is the first study that provides some evidence of the potential benefit of the use of A(1–7) analogue, NLE, as a therapeutic target for acceleration of corneal wound healing with added benefit of reducing scar formation.

**METHODS**

All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the University of Southern California. Rabbits were anesthetized with a subcutaneous injection of ketamine hydrochloride (25 mg/kg) and xylazine hydrochloride (6 mg/kg).

**Animal Groups**

We used 45 Dutch age-matched pigmented rabbits, weighing 2 to 3 kg. Only the right eye of each animal was used for treatment. A total of 15 rabbits were assigned to the safety study (normal, nonoperated cornea; five rabbits for each test material) and 30 rabbits were assigned to the efficacy studies.

**Test Material**

Animals allocated to active treatment groups received one eyedrop of USB004 (0.03% or 0.3% NLE in 2% hydroxyethyl cellulose [HEC] in 0.05 M phosphate buffer, pH 6.5) daily. Animals allocated to the control group received one eyedrop of vehicle (2% HEC in 0.05 M phosphate buffer, pH 6.5) daily.

**Safety Study Design**

Noninvasive study was conducted in 15 normal rabbits (divided into three equal groups) with no incisional wound to evaluate the effect of the test article on the cornea in terms of inflammation or edema in addition to IOP measurements.

Each group received one eyedrop of 0.03% or 0.3% USB004 or vehicle (control) daily for 28 consecutive days. The safety of USB004 was evaluated by IOP measurements using Tonopen-AVIVA (TPA, Reichert, Inc., Depew, NY, USA), slit-lamp (Haag-Streit AG, Gartenstadistrasse, Switzerland) on a weekly basis, and clinical findings were graded using a modified McDonald-Afora grading scale. The mean of the three scans of each cornea (MCT) was used as the final result for corneal swelling. The same masked operator performed all OCT scans during each examination. The measurement of corneal thickness in the corneal apex was assessed using the OCT software “caliper” function. This measurement was repeated in all 12 scanned meridians, between 0° and 165° with 15° intervals.

**Heidelberg Retinal Tomogram (HRT III)**

All animals in the study were subject to quantitative 3-dimensional corneal imaging using HRT-III for both eyes at every follow-up visit. The HRT III (Heidelberg Engineering GmbH) was converted into an in vivo confocal microscope using a Rostock Cornea Module for investigating the ocular surface. Images consist of 384 \(\times\) 384 pixels covering 400 \(\times\) 400 \(\mu\)m with transversal optical resolution of approximately 1 \(\mu\)m/pixel and an acquisition time of 0.024 seconds (Heidelberg Engineering GmbH). The x-y position of the image and the section depth are controlled manually. Before microscopy evaluation, one drop of Tetracaine (oxybuprocaine 0.4%; MSD-Chibret, Paris, France) and one drop of gel tear substitute (Lacrigel, carbomer 0.2%; Europhtha, Monaco) were instilled in the lower conjunctival fornix. Confocal microscopic images of tangential optical sections of the corneal epithelium, superficial stroma, deep stroma, and corneal endothelium were taken. Optical sections were obtained at 150, 250, 350 \(\mu\)m from the epithelial surface.

**Leakage Pressure Test**

One week after the surgical procedure, three animals from each treatment group were assigned to this experiment to

incise Descemet’s membrane. To prevent globe collapse after corneal tunnel creation, a small amount of viscoelastic was injected into the anterior chamber through a paracentesis. This was done only in the first efficacy study as the viscoelastic escaped from the corneal incision after injection. Intramuscular (IM) buprenorphine (Buprenex) 0.01 to 0.05 mg/kg was administered immediately after surgery and 24 hours later for pain management. Gentak (Gentamicin Sulfate Ophthalmic ointment USP, 0.3%; Perrigo, Minneapolis, MN, USA) also was administered to reduce risk of infection.

**Seidel’s Pressure Test for Corneal Leakage**

Corneal wound leakage was tested using a cobalt blue filter slit-lamp after one drop of 10% fluorescein (Akorn, Abit Springs, LA, USA) was instilled in the conjunctival sac and a slight pressure was applied to the globe with a Q-tip applicator. Any changes in color or surface of the fluorescence area indicate the presence of corneal leakage.

**Ocular Examination**

Examinations included evaluation of the anterior segment by slit-lamp biomicroscopy and HRT-III. Baseline examinations were performed immediately before the surgical procedure. Follow-up examinations continued weekly through the 28-day study. Clinical findings at each examination were graded as mentioned previously.
assess the stability of corneal wounds. A 27-gauge needle (BD Biosciences, Franklin Lakes, NJ, USA) was connected to an infusion system with a BSS bag (Abbott Laboratories, Abbott Park, IL, USA) inserted at the 5 o’clock position relative to the surgeon’s view and parallel to the iris plane. BSS bag pressure was adjusted with rigid tubing to a Constellation Vision System (Alcon Laboratories, Inc., Fort Worth, TX, USA). Intraocular pressure was increased in 10 mm Hg increments to determine the highest IOP achievable before any wound leakage was observed. Wound leakage was assessed by painting the wound with fluorescein paper strip fluoresceins (Fluorescein Sodium Ophthalmic Strips USP; Bausch & Lomb, Rochester, NY, USA) and observing any greenish change.

After the final examination, the rabbits were killed by intracardiac injection of 2 ml pentobarbital (Beuthanasia-D; Schering Plough Animal Health, Omaha, NE, USA).

Light Microscopic Examination

Enucleated eyes (both eyes from each animal) were symmetrically sectioned across the corneal wound site, then one-half of each eye was immersed in Davidson’s fixative solution overnight, then dehydrated in a series of graded alcohol solutions over the next 24 to 48 hours before paraffin embedding. Blocks were obtained from cuts through the whole globe oriented perpendicularly to the medullary wings. Sections 5-μm thick, stained with hematoxylin and eosin (H&E) were examined by light microscopy and corneal sections photographed using the same magnification.

Scanning Electron Microscopy (SEM)

The other half of the sectioned eyes were fixed in half-strength Karnovsky’s fixative for 2 days, rinsed with PBS and fixed with 1% osmium tetroxide in 0.1 M PBS for 1 hour. Specimens were dehydrated using a series of graded ethyl alcohol solutions and chemically dried using hexamethyldisilazane. Specimens were mounted on aluminum stubs with adhesive tabs and sputter coated for 3 minutes (Polaron; Energy Beam Sciences, Agawam, MA, USA), then viewed with a scanning electron microscope (ISM-6390; JEOL USA, Inc., Peabody, MA, USA).

Statistical Analysis

All analyses were conducted using the SPSS 19.0 statistical package (SPSS, Inc., Chicago, IL, USA) to calculate means and standard deviation. Analysis of variance (ANOVA) or χ² analyses were used when appropriate to compare various parameters between the vehicle versus USB004-treated animals. Statistical significance was a priori set with an α of 0.05 for statistical significance.

RESULTS

Safety of USB004 in Healthy Rabbit Eyes

No significant IOP changes were detected following repeated administration of 0.3% or 0.03% USB004 when compared to vehicle control. In the 0.3% USB004, a mean of 15 mm Hg (range, 12–15 mm Hg) IOP was recorded at baseline. Repeated administration of 0.3% USB004, 0.03% USB004, or vehicle showed no significant change in mean IOP measured at 12.6 (range, 12–13.2), 13 (range, 12–13.2), and 12.7 (range, 12.6–13) mm Hg, respectively. There were no abnormal slit-lamp observations or histopathologic signs of inflammation or toxicity.

Clinical Evaluation After Corneal Incision

Wound edema was observed only in the first 7 days in the USB004 treatment groups, in contrast with the vehicle-treated group where edema was noted up to 21 days. Corneal edema extending beyond the wound margins was more marked in the vehicle treatment compared to the USB004-treated groups. No postoperative infection was reported. Conjunctival congestion was marked the first 2 weeks in the vehicle group (grade II) compared to 1 week for the USB004-treated groups (grade I).

Wound closure was assessed using Seidel’s test with pressure. A positive Seidel’s test was observed on day 3 for all treatments. However, a negative Seidel’s test was achieved for animals treated with 0.03% or 0.3% USB004 from day 7 onwards for the first five rabbits in each group (first efficacy study) in contrast to day 7 for the vehicle-treated animals. For the subsequent five rabbits (second efficacy study), leakage was not observed at day 5 for the 0.3% and 0.03%-treated animals in contrast to day 11 for the vehicle-treated animals.

HRT-III and Anterior Segment OCT Results

Average MCTs, an indicator corneal edema severity, as measured by SD-ASL at the center of the corneal wound, were statistically significant when comparing vehicle with USB004 treatment groups at the final examination (P < 0.001; Fig. 1). At the final examination, there was no clinically significant difference between MCT in 0.03% or 0.3% USB004-treated eyes when compared to baseline levels. However, statistical increase in MCT was observed in vehicle-treated animals at weeks 1 to 3 when compared to eyes that were treated with either 0.03% or 0.3% USB004. Our data also showed the coefficient of variation (CV) to be less than 2% in all study groups during the 4-week follow-up period (Fig. 2).

Stromal architecture was evaluated using HRT-III. The vehicle-treated control group showed marked disorganization of the collagen lamellae. In contrast, 0.3% and 0.03% USB004-treated eyes showed smooth, regularly arranged lamellae at the end of the follow-up period (Fig. 3). Images were acquired from the central cornea at the following depths: epithelium, subbasal nerve plexus (immediately anterior to Bowman layer), anterior stroma (up to 150-μm depth), mid stroma (250-μm depth), posterior stroma (350-μm depth), and endothelium. Endothelial cell counting (ECC), mean cell area (MCA), and polymorphism (percentage of hexagonal cells) were measured at the final time point (Fig. 3).

Epithelium. Closure of the epithelial wound typically occurred within 4 days in the USB004 treatment groups. Slight
Subepithelial haze in the reformed epithelium was visible at 1-week follow-up, but largely dissipated by 1 month. At the final examination, the epithelium in treatment groups appeared to be largely unaffected by surgery, whereas the vehicle-treated group showed a disrupted epithelium.

**Subbasal Tarsal Plexus.** The subbasal nerve plexus was essentially absent at 1 week in all three treatment groups. The subbasal nerve plexus appeared to regenerate by the end of week 2 after treatment with 0.03% and 0.3% USB004; only partial regeneration was seen after 1 month in the vehicle-treated group.

**Stroma.** In the USB004 treatment groups, keratocyte density and reflectivity appeared to be largely unaltered when compared to nonsurgical animals at the final postoperative examination (Fig. 3). This is in contrast to the vehicle-treated eyes, where keratocyte density and reflectivity were significantly altered by surgery. At 1 week postoperatively, all the study groups showed complete absence of stromal keratocytes, increased tissue reflectivity, and a honeycomb-like appearance. The anterior stroma had a similar appearance at 2 and 3 weeks, but at 1 month there was evidence of keratocyte repopulation in the treatment groups, with brighter cells possibly representing active keratocytes (fibroblasts).

**Endothelium.** At the final examination, quantitative and qualitative analysis of endothelial cell densities confirmed differences \( P < 0.001 \) between the vehicle-treated groups compared to nonsurgical animals at the final postoperative examination (Fig. 3). This is in contrast to the vehicle-treated eyes, where keratocyte density and reflectivity were significantly altered by surgery. At 1 week postoperatively, all the study groups showed complete absence of stromal keratocytes, increased tissue reflectivity, and a honeycomb-like appearance. The anterior stroma had a similar appearance at 2 and 3 weeks, but at 1 month there was evidence of keratocyte repopulation in the treatment groups, with brighter cells possibly representing active keratocytes (fibroblasts).
MasR activation also can induce cells to differentiation into specialized cells which is based on the proliferation of keratocytes, fibroblasts, and microvascular endothelial cells from injured tissue. 33, 37, 38, 42–44 Our group previously showed that MasR agonists stimulate progenitor cells to upregulate expression of RAS receptors. 37, 37, 48 These receptors are downregulated after wound resolution. 47 One of the RAS receptors that is upregulated is MasR, a G-coupled protein receptor found on the surface of keratinocytes, melanocytes, fibroblasts, and microvascular endothelial cells from injured tissues. 46

and USB004-treated groups (see Table). Polymorphism was significantly more marked with the vehicle than in the USB004 treatment groups. A demarcation line is visible between the anterior hyperreflective stroma and posterior stroma with normal reflectivity.

**Leakage Pressure Test Results**

Assessment of wound stability showed pressure leakage 79.3 ± 6.0 mm Hg for the 0.3% USB004 group and 77.3 ± 3.2 mm Hg for the 0.03% USB004 group compared to 38.7 ± 3.5 mm Hg for the vehicle control (P < 0.001). These findings suggest that USB004 promotes a more stable wound when compared to vehicle control (Fig. 4).

**Hematoxylin and Eosin Staining**

Vehicle-treated corneas showed signs of incisional injury; the corneal stromal was disorganized with evidence of cellular infiltration and fibrosis found throughout the length of the incision. In contrast, sections from USB004-treated corneas showed regularly-arranged stromal lamellae, regular alignment of the corneal endothelium, and near-normal corneal thickness (Fig. 5).

Cellular infiltration was reduced with 0.03% USB004, while infiltration was undetectable in eyes treated with 0.3% USB004 (Fig. 5). Fibrosis was found throughout the length of the incision in vehicle-treated cornea, in contrast to 0.3% USB004-treated (Fig. 5, left) eyes. Treatment with USB004 showed a concentration-dependent healing, where no signs of fibrosis or cellular infiltration were seen in eyes treated with 0.3% USB004. Throughout the healing process, no blood vessel formation was observed.

**Electron Microscopy**

Scanning electron microscopy was used to evaluate the impact of various treatments on wound morphology. Cracking of the corneal wound site was useful in studying internal structure at the healing zone. Scanning electron microscopy, with magnification increasing from ×300 to ×2300, verified findings of light microscopy, as well as rarefaction of the collagen lamellae in the control group compared to the more compact architecture seen in the treatment groups (Fig. 6).

**DISCUSSION**

Angiotensin peptides have been demonstrated to modulate cellular proliferation, angiogenesis, and dermal repair. 46–48 We have shown previously that MasR agonists stimulate progenitor cells to differentiation into specialized cells which is based on tissue requirements. 35, 37, 38, 42–44 MasR activation also can oppose AII-mediated inflammation and fibrosis. 32, 45 Furthermore, MasR expression has been detected in the cornea using immunostaining and A(1–7) localization in the basal and superobasal epithelial cells. 46

Our group previously showed that NLE administration can accelerate epithelial wound healing. 37, 38 Topical or systemic administration of NLE reduced fibrosis and scarring in the healing wounds. This action was more pronounced with longer administration of the peptide after injury. The action of this peptide was blocked by the MasR antagonist, further suggesting that activation of MasR is involved in the healing responses to exogenous NLE administration. Administration of NLE during the remodeling phase of wound healing reduced scar formation by acceleration of epithelial healing, extracellular matrix deposition, and keratocyte proliferation. 37

The active pharmaceutical ingredient of USB004 is NLE, an analog of A(1–7), a natural ligand for the MasR. In this study, the ocular application of 0.3% or 0.03% USB004 was found to be safe in healthy and incised eyes, where no significant changes in IOP or signs of inflammation were noted when compared to preincision levels.

Treatment with 0.3% and 0.03% USB004 can accelerate full-thickness corneal wounds in a concentration-dependent manner. This was associated with rapid resolution of edema, reduced inflammation, and reduction in wound leakage duration. USB004-treated incisions demonstrated a consistent near-normal architecture without evidence of fibrosis when compared to vehicle-treated animals. The corneal efficacy was consistent across the treatment groups where the CV was >2% with no outliers in any of the treated eyes. These findings are consistent with the pharmacologic activity of this heptapeptide, which accelerates dural healing and reduces scar formation. 38, 47, 48

Immediately following tissue injury, cells on wound edges upregulate expression of RAS receptors. 37, 37, 48 These receptors are downregulated after wound resolution. 37 One of the RAS receptors that is upregulated is MasR, a G-coupled protein receptor found on the surface of keratinocytes, melanocytes, fibroblasts, and microvascular endothelial cells from injured tissues. 46

**FIGURE 4.** Pressure required to induce incisional leakage. The amount of pressure required to induce full-thickness leakage between vehicle only, and the two concentrations of USB004 (0.03% and 0.3%). Significant difference was detected between vehicle and USB004 treatment (P < 0.001), but no difference was demonstrated between the two USB004 concentrations.
tissues. MasR agonists promote wound-healing, as clinically demonstrated in patients with diabetic foot ulcers.\textsuperscript{48} USB004 may use the same mechanism(s) to accelerate full-thickness corneal wound healing. Previous results suggest that USB004 can stimulate recellularization of cutaneous wounds by promoting the regeneration of precursor stem cells from the epidermal niche.\textsuperscript{38,47} It is hypothesized that USB004 also may use these mechanism(s) to enhance proliferation and differentiation of cornea-specific stem cells located in the limbal niche (Fig. 3).

Confocal microscopy showed that cellular organization in USB004-treated eyes had morphology similar to that of normal tissues. This was supported further by incisional closure strength, which was higher in USB004-treated eyes than vehicle-treated eyes (Fig. 4). Level of fibrosis was evaluated with SEM, showing that USB004 treatment reduced rarefaction of the collagen stroma lamella (Fig. 6).

The lack of wound fibrosis found with USB004 treatment is consistent with MasR activation, which may be due to its ability to prevent AII-induced fibrosis.\textsuperscript{49} Profibrotic mechanisms can be activated through AII binding onto AT1R.\textsuperscript{50} Stimulation with AT1R leads to activation of ERK1/2 and TGF-\beta pathways that can in turn promote phosphorylation of Smad2/3, which can then activate CTGF, a profibrotic regulator. Stimulation of MasR can prevent AII-induced phosphorylation of ERK1/2 and Smad2/3, and, thus, block TGF-\beta pathways.\textsuperscript{51–53}

**FIGURE 5.** Hematoxylin and eosin-stained cornea slides in vehicle, 0.03%, and 0.3% USB004. (A–C) Magnification: ×20. (D–F) Magnification: ×40. In vehicle-treated cornea there was marked stromal edema, cellular infiltration, and tissue disorganization (A, D). A concentration-dependent ([B, E] 0.3% USB004, and [C, F] for 0.03% USB004) showed accelerated wound healing in a concentration-dependent manner. Cornea from 0.3% USB004 showed a resolution of wound with the lack of blood vessels and cellular infiltrates.

**FIGURE 6.** Photomicrographs of SEM of corneal sections from vehicle, 0.03%, and 0.3% USB004 treatment in full-thickness corneal injury. Left to right: magnification from ×300, ×600, and ×2300 of the treatment. Marked rarefaction of the collagen stromal lamellae in the vehicle-treated control group when compared to either 0.03% (middle panel) or 0.3% USB004 (lower panels).
Thus, the ability of USB004 to activate MasR may explain how it can accelerate wound healing without fibrosis in the full thickness corneal incision.

These studies suggest that treatment with USB004 accelerates incisional wound closure that is robust and durable. Microscopic examination of the healed wound showed that the cellular architecture was organized and indistinguishable from nonwound areas. Histologic and SEM findings did not reveal any signs of fibrosis. One key element in the USB004-mediated accelerated closure was the lack of blood vessel formation, which suggests that USB004 is able to promote avascular healing. The angiogenic activities of Mas agonist were demonstrated previously in xenograft prostate cancer models where VEGF and placental growth factor (PIGF) were shown.54

Our data showed that USB004 promotes avascular corneal healing in full-thickness wounds. Our study has a few limitations, namely the small size of the study groups and lack of immunohistochemical studies using specific antibodies against A(1–7)/MasR. Hence, further corneal specific investigations to determine the precise molecular mechanisms driving USB004’s activities are required to address these limitations.

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