Title: Connexin43 Modulation Inhibits Scarring in a Rabbit Eye Glaucoma Trabeculectomy Model

Running title: Inhibiting Scarring in a Rabbit Glaucoma Surgery Model

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Abstract

We investigated whether one subconjunctival injection of connexin43 antisense oligodeoxynucleotides (Cx43 AsODN) modulates postoperative scarring in a rabbit model of glaucoma trabeculectomy surgery. In a randomized, controlled, masked-observer study, 39 rabbits underwent trabeculectomy surgery with insertion of a 22 gauge cannula in the right eyes and were randomly divided into 3 treatment groups. Each rabbit received an injection of Cx43 AsODN in Pluronic gel, BSS or Pluronic gel alone into the formed bled. The animals were euthanized at 8 hours, 24 hours, 5 days and 21 days. Histology and immunohistochemistry results demonstrated that Cx43 AsODN decreased Cx43 upregulation at 8 and 24 hours which led to less myofibroblast upregulation at day 5 and 21 and reduced scarring at day 21 compared to controls. We conclude that postoperative use of Cx43 AsODN inhibited subconjunctival scarring and fibrosis. Cx43 AsODN injection may be a safe and effective anti-scarring agent in glaucoma trabeculectomy surgery.

Key words: Scar Reduction, Gap Junction, Connexin43, Glaucoma, Trabeculectomy

Abbreviations: 5-FU, 5-fluorouracil; α-SMA, alpha smooth muscle actin; BSS, balanced salt solution; Cx43, connexin 43; Cx43 AsODN, connexin43 antisense oligodeoxynucleotides; IOP, intraocular pressure; and MMC, Mitomycin-C.

Introduction

Glaucoma is a progressive optic neuropathy and the second leading cause of irreversible blindness in the world [1]. When topical medical therapy and/or laser treatment are inadequate, surgical intervention is used to improve aqueous fluid egress. Trabeculectomy is the most widely used incisional surgery to enhance aqueous drainage, involving the removal of partial thickness limbal tissue containing, trabecular meshwork, sclera and cornea under a lamellar scleral flap [2, 3]. The surgery results in an internal sclerostomy that is partially covered with a flap of tissue from the sclera and the conjunctiva. As the fluid flows through the new drainage opening, the tissue over the opening rises to form a blister in the subconjunctival space, called a bleb. The primary mechanism by which a trabeculectomy lowers intraocular pressure (IOP) is via subconjunctival filtration with subepithelial microcysts forming channels for the passage of aqueous [4]. The aqueous exits across the conjunctival epithelium into the tear film, or by direct absorption into blood vessels in the subepithelial connective tissue [5].

There is consensus that when there is an elevation of IOP following trabeculectomy, it is due to an exaggerated postoperative wound healing response which restricts the flow of aqueous across the conjunctiva [4]. Persistent inflammation around the bleb is a sign of impending failure [6]. In failed blebs, histopathological analysis shows abnormally thickened, dense collagenous connective tissue with visible fibroblasts and blood vessels beneath a normal conjunctival epithelium [5, 7].

In order to to minimize inflammation [8], anti-proliferative agents, 5-fluorouracil (5-FU) and Mitomycin-C (MMC), have become the backbone of anti-scarring treatment by inhibiting fibroblast replication and function [9, 10]. Both agents are associated with significant side-effects such as bleb leaks [11], endopthalmitis [12], hypotony (42%), and hypotony related maculopathy (8.9%) [13]. Though effective, therefore, these anti-proliferative agents are a double edged sword, with complications linked to their lack of specificity. There is a drive to develop an adjuvant to improve trabeculectomy success rates that has localized, predictable action, ease of administration and a minimal side effect profile. Modulation of gap junction communication following surgery is one possibility.

Gap junctions are structures that allow direct signalling between cells [14]. Six connexin protein subunits oligomerize to form a hemichannel called a connexon; two connexons from neighboring cells dock to form a complete intercellular junction channel. Multiple intercellular channels cluster together to form gap junctions with the number of channels in a plaque varying considerably [14]. Connexin43 (Cx43) is one of the most ubiquitous and more

studied isoforms [15-17]. Gap junctions play a role in inflammation [18-20], cell migration [21, 22] and tissue contraction [23]. Rodent studies demonstrate that a transient reduction in Cx43 protein expression is beneficial in skin wound healing and scar reduction [24, 25]. These studies used connexin43 antisense oligodeoxynucletides (Cx43 AsODN) to cause a transient knock down of Cx43 in incision, excision and burn skin wounds. A knockdown of Cx43 expression was achieved at two hours and maintained for 24 hours, resulting in reduced edema, reduced inflammation and an improved rate of healing. There was decreased neutrophil migration into the wound site with knockdown of Cx43 in rat skin [25] with a subsequent reduction in inflammatory cytokines and the number of macrophages [26]. The initial down-regulation is reported to have lasting effects with smaller areas of granulation tissue deposition in the treated groups compared to controls [25]. A similar reduction in inflammation (reduced neutrophil infiltration, astrocytosis, microgliosis and vascular disruption) has also been reported with Cx43 AsODN application following rat spinal cord and optic nerve injury [27, 28].

We hypothesized that subconjunctival application of Cx43 AsODN gel at the time of surgery in a rabbit trabeculectomy model would cause transient down regulation of Cx43 protein expression resulting in decreased inflammation and reduced deposition of scar tissue.

Methods

New Zealand Albino White Rabbit Trabeculectomy Glaucoma Surgery Model

Ethical approval was obtained for all animal experiments from the Auckland University Animal Ethics Committee. All animals were treated in accordance with the University of Auckland Animal Ethics Committee guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animal procedures were carried out in the Vernon Jansen Animal Research Unit at the University of Auckland. The right eye of each animal was operated on. The left eye was used for comparison during analysis.

A modified trabeculectomy procedure was used [29] with a cannula to maintain a patent scleral tract. Intramuscular injection of Ketamine (100 mg/ml, Parnell Laboratories New Zealand Limited) at a dosage of 0.35 mg/kg and Xylazine (20 mg/ml, Phoenix Pharm Distributors Ltd) at a dosage of 5 mg/kg was used to anesthetize the animal. This was complemented by the administration of a single drop of a topical anesthetic - Benoxinate 0.4% (Chauvin Pharmaceuticals) on the eye.

A lid speculum was inserted to expose the bulbar conjunctiva and a drop of iodine was instilled and washed out with a balanced salt solution (BSS, Alcon Laboratories). Two partial thickness 6-0 silk corneal traction sutures were placed superiorly and the eye pulled inferiorly. A conjunctival incision was then made supero-temporally, approximately 10 mm from the limbus. A limbal based conjunctival flap was raised with blunt dissection of the subconjunctival space to the limbus using Westcott scissors. Any bleeding encountered was controlled with application of pressure and, if needed, thermal cautery. A micro vitreoretinal blade was used to fashion a partial thickness scleral flap approximately 4 mm from the limbus. The tunnel acted as a starting point for the 22 gauge 25 mm Venflon intravenous cannula (BD Biosciences) which was passed anteriorly until the cannula needle was visible in the cornea. Entry into the anterior chamber was then made with the cannula needle. The cannula was advanced to the mid-pupillary area, away from the iris, and the needle withdrawn. The scleral end of the cannula was then trimmed 1 mm from the scleral insertion point and fixed to the sclera with a 10-0 nylon suture. The conjunctival incision was closed with a continuous 10-0 nylon suture. The anterior chamber was reformed with BSS and the conjunctival incision evaluated for water-tightness using fluorescein.

At the appropriate time point animals were euthanized using Pentobarbitone (30 mg/kg) administered intravenously through the ear vein using a 22G needle. Eyes were then enucleated. Starting with a lateral canthotomy, the lids were everted and a superior tarsal conjunctival incision made and from this incision circumferential peritomy was performed, avoiding the bleb area. The extra-ocular muscles were identified and cut. The inserted ends of the extra-ocular muscles were used to pull the eye forward, allowing blunt dissection to the optic nerve. The optic nerve as well as the extensive retro-orbital venous plexus was cut and the eye removed.

Cx43 AsODN and Delivery

An unmodified Cx43 AsODN sequence of 30 bases long was used as previously described [25, 30, 31]. The rabbit specific antisense oligodeoxynucleotide was 5’ - GTAATTGCGGCAAGAAGAATTGTTTCTGTC - 3’ and was delivered in ice cold Pluronic F-127 gel (30% w/v in PBS, BASF). To achieve this 150 µL of either 10 µM or 50 µM Cx43 ASODN in Pluronic gel was loaded into pre-chilled 1mL tuberculin syringes with a 27G needle. Two types of controls were used; 150 µL of BSS and 150 µL of Pluronic gel alone. Animals were randomly allocated in a masked fashion into one of the three groups. Cx43 AsODN, Pluronic gel or BSS was injected directly into the formed bleb at the end of the procedure. At the end of the procedure the animals received a single dose of

Chloramphenicol antibiotic eye drops (Alcon Laboratories) and Pred Forte anti-inflammatory eye drops (Allergan Inc.).

Stability of Cx43 AsODN in vitro

The stability of Cx43 AsODN in the delivery vehicle has been well established [24, 25, 30, 32]. However, to ensure no breakdown of Cx43 AsODN in the tissues and compounds that it would come into contact with during this study, polyacrylamide gel electrophoresis analysis was carried out. The stability of Cx43 AsODN was evaluated in BSS, rabbit basal tear film, reflex tear film, aqueous, vitreous, Benoxinate anesthetic eye drops, fluorescein, Chloramphenicol antibiotic eye drops, and Pred Forte anti-inflammatory eye drops.

Cx43 AsODN (10 µM final concentration) was combined with each substance and incubated for one hour at 37°C. Samples were then heated to 95°C for 2 minutes to denature any possible secondary structures and immediately chilled on ice for loading onto a 15% acrylamide / urea gel. Bromophenol blue was loaded in one lane only and acted as a molecular weight marker as it runs at the same level as a 9-10 base oligodeoxynucleotide. Gels were run at 200 mV for 35 minutes and the oligodeoxynucleotides in the gel then stained with 0.2% methylene blue for 30 minutes. The gel was de-stained with water and imaged using a Canoscan 8800F scanner.

Immunohistochemistry and Histology

To map Cx43 AsODN effects on Cx43 changes after trabeculectomy, animals were euthanized at 8 hours and 24 hours following surgery and tissue processed for cryosectioning (3 BSS and 3 Cx43 AsODN treated animals at 8 hours, two animals each at 24 hours). The surgical site was excised immediately following enucleation and fixed in 4% paraformaldehyde for 1 hour. The tissue was washed 3 times, 15 minutes each in PBS, embedded in optimal cutting temperature compound (IA018; ProSciTech, Queensland, Australia) and frozen in liquid nitrogen. The cannula was left *in situ* until just prior to freezing. Cryosections were made across the lumen of the cannula path.

A mouse monoclonal antibody specific to the Cx43 protein (from Dr David Becker, University College London, London) was used at 1:1000 dilution with overnight incubation at 4°C. A goat anti-mouse secondary antibody conjugated to CY3 (Jackson ImmunoResearch Laboratories, PA) was used. The nuclear marker DAPI (4', 6-diamidino-2-phenylindole, Invitrogen, Carlsbad, CA) was applied to all sections to provide anatomical orientation.

Images were collected using a Zeiss LSM510 confocal laser scanning microscope. Images were acquired using a x40 objective, with serial optical slices at 1 µm intervals taken in the z plane over 15 µm depth. Z stacks were maximally projected to allow for Cx43 spot counts using ImageJ software version 1.43 (National Institutes of Health, Bethesda, MD) from a volume of 230 µm x 230 µm x 15 µm for areas immediately either side and above or below the cannula. Cx43 protein expression at 500 µm, 1000 µm and 1500 µm either side of the cannula site in control and Cx43 AsODN treated trabeculectomies were assessed with three montage stacks to cover greater conjunctiva – sclera thickness (total volume 690 µm x 230 µm x 15 µm). The corneal epithelium is known to express Cx43 and was used as a positive control tissue.

All other specimens were processed for paraffin sectioning. At 5 and 21 days myofibroblast levels were analysed (at 5 days: 3 BSS and 3 at 10 µM Cx43 AsODN; at 21 days: 11 controls - 9 BSS and 2 gel only and 9 Cx43 AsODN – 6 at 10 µM and 3 at 50 µM) and at 21 days scar tissue was analysed. For paraffin processing the surgical site was carefully excised from enucleated eyes, preserving the conjunctiva and the gross bleb morphology and fixed in 4% PFA for 24 hours. Samples were processed for automatic paraffin embedding through a vacuum tissue infiltration processor (Tissue Tek V.I.P, Sakura Finetek, USA). Following infiltration, the cut tissue was mounted in paraffin blocks (Shandon Histocentre 2, Thermo Electron Corporation, USA) so that sections along the cannula longitudinal axis could be taken for analysis. Five µm thick paraffin sections were cut with the cannula *in situ* and immunohistochemically labeled with a mouse anti - Alpha Smooth Muscle Actin antibody (α-SMA) (1:50; NCL-SMA, Novocastra) followed by goat anti-mouse seccondary antibody conjugated to CY3 (Jackson ImmunoResearch Laboratories, PA), or stained with hematoxylin and eosin. The α-SMA immunoreactivity was used to indicate the presence of myofibroblasts [33] and immunoreactivity was ranked by a masked observer as none, little or extensive. For scar assessment in hematoxylin and eosin stained sections, two parameters were graded - cellularity and density of tissue adjacent to the cannula site. A four tiered grading system was used for each parameter (maximum score 8) with two independent masked observers: 1 = 1-25% more cells or tissue density than normal, 2 = 26-50% more than normal, 3 = 51-75% more than normal, 4 = 76% to 100% more than normal. Mean scores were calculated for each treatment group. Sections were also stained with Picrosirius Red and viewed under polarized light to assess the amount of collagen present.

Assessment of Clinical Parameters

Postoperatively, all animals were reviewed daily for the first five days and then every 3-5 days thereafter until 21 days. In this group there were 12 controls - 9 BSS and 3 gel only and 9 Cx43 AsODN – 6 at 10 µM and 3 at 50 µM. The observer was masked as to which group the animal belonged to. Bleb presence, bleb morphology, anterior chamber reaction and corneal pathology were assessed using a slit lamp whilst the rabbits were wrapped in a towel. The bleb was photographed with a scale bar and bleb area measured using ImageJ.

Rabbits are known to have a significant anterior chamber reaction postoperatively and it was assessed using the slit lamp. Anterior chamber reaction manifested with the presence or absence of fibrin with flare. A two tiered grading system was used: 0 = quiet or 1 = fibrin. Eyes where a residual strand of fibrin was seen only within the lumen of the cannula were considered as quiet.

Intraocular pressure was assessed after the administration of topical anaesthetic drops (Benoxinate 0.2%) to each eye every day for 5 days and every three days thereafter using a Tonopen. Baseline IOP measurements were taken in each eye prior to surgery. An average of three readings with <5% error was taken as the final reading in each eye. The Tonopen was re-calibrated at the beginning of each clinical assessment day.

Results

Stability of Cx43 AsODN in Vitro

Whilst the stability of the human sequence of Cx43 AsODN has been established in its delivery vehicle, Pluronic F-127 gel [24, 25, 30, 32], it had not been previously trialled in rabbits or with the pharmaceutical reagents that would be used concomitantly with any surgical protocol. Cx43 AsODN (10 µM) was incubated with New Zealand white rabbit basal tear film, reflex tear film, aqueous and vitreous and evaluated for evidence of product breakdown using gel electrophoresis. The same protocol was also carried out with Benoxinate eye drops, Chloramphenicol eye drops, Pred Forte eye drops, and BSS. There was no evidence of Cx43 AsODN breakdown with any of these substances indicating that the Cx43 AsODN was stable in the experimental environment it was to be used in (Figure 1).

Cx43 Expression in the Rabbit Sclera and Conjunctiva

In uninjured rabbit eyes some Cx43 immunoreactivity was evident in the basal layers of the conjunctiva (Figure 2A) but labeling was limited in comparison to that in the basal layers of the corneal epithelium used as a positive control (Figure 2B). There was virtually no Cx43 immunoreactivity in uninjured sclera and little Cx43 immunoreactivity in the subconjunctival tissue.

Acute Changes in Cx43 Expression

Eight hours following injury there was significant upregulation in Cx43 expression adjacent to the cannula in the subconjunctival tissue which is located between the conjunctiva and the sclera (Figure 3). This increase in Cx43 expression following trabeculectomy was significantly attenuated ($p<0.05$) in the 10 µM Cx43 AsODN treated wounds. Cx43 expression was quantified (number of labeled spots per image field) at 500 µm, 1000 µm and 1500 µm either side of the cannula site and was signifcantly greater at all three distances ($p<0.05$) on either side of the cannula in the BSS treated eyes compared to the Cx43 AsODN treated eyes (Figure 4). A similar Cx43 immunoreactivity pattern was still evident in the subconjunctiva at 24 hours post-injury but to a lesser degree than at 8 hours (data not shown).

Myofibroblast Level and Scar Tissue Assessment

Animals were culled at 5 and 21 days post trabeculectomy and processed as paraffin sections to evaluate the presence of myofibroblasts at that time point. An antibody against α-SMA which labels the actin microfilaments in myofibroblasts was used and labeling of blood vessel walls acted as a positive control within each section (Figure 5). Overall the Cx43 AsODN treated animals showed decreased expression of α-SMA compared to BSS control specimens 5 days following trabeculectomy. Masked assessment of the specimens at 21 days demonstrated that 36% (4 out of 11) of control eyes showed extensive α-SMA labeling at the surgical site, while only 11% (1 out of 9) of Cx43 AsODN treated eyes showed equivalent levels of α-SMA.

There were marked differences in hypercellularity and scar tissue deposition between Cx43 AsODN treated eyes and controls at 21 days following trabeculectomy demonstrated using hematoxylin and eosin and picrosirius red staining (Figure 6). There was no observable difference between BSS and Pluronic gel treated eyes, therefore these groups were combined during analysis and simply called ‘control’. In the control eyes a strong cellular

response remained and there was a denser packing of collagen fibers. The scar tissue in the control eyes contained densely packed cellular infiltrate often extending into the lumen of the cannula and subconjunctival tissue appeared firmly adherent to the sclera with no fenestrations. Picrosirius red staining viewed under polarized light shows collagen fibres in different colours according to the diameter or thickness of the fibres, their density of packing and the extent of alignment [34]. Smaller diameter, loosely packed collagen fibres appear green or yellow; larger diameter and more tightly packed collagen fibres appear orange or red [35]. In the Cx43 AsODN treated eyes a reduced cellular response was noted and the collagen was less densely packed with decreased intensity of labelling. The scar tissue present still tended to occlude the mouth of the cannula but there was minimal scarring within the lumen of the cannula itself. The subconjunctival tissue was more loosely adherent to the sclera and fenestrations, as a result of aqueous drainage, were often visible within the subconjunctiva tissue. Ranking of the cellular response and scar tissue deposition by two masked observers gave rank score averages for the control operated eyes 5.7 (out of 8) and for the Cx43 AsODN treated eyes 3.8 (out of 8) with no difference between 10 μM and 50 μM dose ($p = 0.42$). There was good inter-observer agreement with a kappa coefficient of 0.81 (with a 95% confidence interval of 0.70 - 0.91) and the difference between Cx43 AsODN treated and control eyes was significant ($p = 0.04$). Scar tissue was evident within the cannula lumen in 72% of control animals but in only 25% of those treated with Cx43 AsODN.

Clinical Parameters

Bleb areas initially appeared larger in Cx43 AsODN treated eyes but all blebs failed by day 12 post surgery. Median survival time for blebs in the treated group was 9 days and the control group 7 days but there was no significant difference between groups. All animals had fibrin with associated flare within the anterior chamber through until at least 3 days post procedure but the Cx43 AsODN treated animals resolved this postoperative inflammation significantly sooner (median of 5 days for AsODN treated vs. median of 8 days in controls) ($p = 0.026$) (Figure 7). None of the animals exhibited corneal epitheliopathy at any time point. Intraocular pressure measurements fluctuated with variation up to 11 mm HG even in control unoperated eyes between consecutive days. There was no statistically significant difference between treated and control eyes.

Discussion

The findings of the present study support the idea that modulation of Cx43 expression has therapeutic potential in the decrease of postoperative scarring associated with failed trabeculectomy. We identified that a single injection of Cx43 AsODN reduced the level of myofibroblast activity at 5 and 21 days. The most marked effect was a significant reduction in cellular response at 21 days with less deposition of scar tissue. In untreated eyes scar tissue was deposited within the lumen of the cannula; however, none of the eyes treated with Cx43 AsODN showed any major deposition of scar tissue within the cannula lumen.

Histologically, the reduction in cellular and tissue density in the trabeculectomy area after Cx43 AsODN treatment was comparable to those achieved with MMC [29, 36-38], bevacizumab [39], suramin [37], CAT-152 [40], trehalose [41], and ilomastat [38, 42]. 5-FU seems to have little scar inhibitory effects when compared to bevacizumab [39] and CAT-152 [40]. The high cellular and tissue density seen in our control animals was comparable to that seen in the control animals in the aforementioned trabeculectomy studies.

In rodent and human skin Cx43 is the predominant connexin in lower epidermal layers and in the dermis [43, 44]. Blocking the expression of Cx43 with Cx43 AsODN after cutaneous injury has been shown to restrict edema, inflammation and scarring, and to promote faster wound closure [24-26]. Wounds that are slow to heal, demonstrated *in vivo* in the STZ diabetic rat, may not down regulate Cx43 at the wound edges [45, 46]. Reducing Cx43 upregulation after injury has also been shown to reduce inflammation after spinal cord injury [30, 48] and optic nerve ischemia *ex vivo* [28]. The concept of modulating gap junctions in order to modify the wound healing response is a novel approach which has not previously been applied to glaucoma surgery.

Animal models of glaucoma surgery require either the use of a cannula or the creation of an internal sclerostomy. The use of a cannula in this study to form the sclera tract as opposed to a sclerostomy procedure has both advantages and disadvantages. It has been demonstrated that statistically significant higher levels of the inflammatory markers connective tissue growth factor (CTGF) and Transforming growth factor – beta2 (TGF-β2) are found in animals following the sclerostomy procedures compared to those following the cannula procedure [48]. The cannula model provides a relatively easy procedure and it has been used previously to assess novel anti-scarring agents. Furthermore, processing of the surgical site with the cannula *in situ* increased the accuracy of the histological assessment.

The Cx43 AsODN proved to be stable in eye fluids and pharmaceutical reagents used, and Cx43 protein levels were significantly reduced either side of the cannula, leading to a

significant reduction in cellular response and scarring. The main disparity with our results compared to other approaches used previously though was in the assessment of clinical parameters where better success has been reported with bleb presence and prolonged bleb survival in treated animals [39, 41, 43, 50-52]. A possible explanation is that our surgical technique involved only one injection of Cx43 AsODN at the time of surgery. Other groups often used multiple injections during the postoperative course [38, 41, 47, 48]. For example, a study on the effects of the matrix metalloproteinase inhibitor ilomastat involved daily subconjunctival injections of ilomastat for the first 9 days, twice weekly injections for the following 2 weeks, and once weekly injections for the next 2 weeks [38, 42]. It is possible that repeat deliveries of the Cx43 AsODN may be even more efficacious and this should be attempted in future experiments.

Interestingly, there was evidence of continued chronic inflammation (hypercellularity) at 21 days in both Cx43 AsODN treated and control eyes albeit this was significantly less in the Cx43 AsODN treated eyes. It is possible that the nictitating membrane rubbing on the cannula causes additional chronic irritation to surrounding tissue. The wound healing response in the rabbit compared with that in humans is, however, known to be more aggressive and exaggerated and to entail routine failure of trabeculectomy surgical sites within a few days to weeks [49]. One possible reason may be the intense anterior chamber reaction that can occur. Large fibrin clots have been previously reported in rabbit trabeculectomies at two days post procedure [49]. We are unaware of other studies that specifically commented on the presence of anterior chamber fibrin. One of our primary clinical endpoint parameters was anterior chamber inflammation. Fibrin from the anterior chamber may not only enter and block the lumen of the cannula, but might then also act as a scaffold for inflammatory cells to migrate into the cannula and for scar tissue deposition. In the present study both control surgical eyes and Cx43 AsODN treated eyes showed fibrin with associated flare within the anterior chamber through until at least day 3 post procedure although the Cx43 AsODN treated animals resolved this postoperative inflammation significantly sooner.

In conclusion, a single subconjunctival application of Cx43 AsODN at the time of surgery brought about a transient down regulation of Cx43 protein expression, significantly reduced anterior chamber reaction, reduced levels of subconjunctival myofibroblast activity and decreased deposition of scar tissue, particularly within the lumen of the cannula. Cx43 AsODN shows promise as a non-toxic, physiologically acceptable reagent in reducing the scarring response following trabeculectomy and further studies appear to be warranted.

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FIGURE LEGENDS

Figure 1: Polyacrylamide gel electrophoresis analysis of Cx43 AsODN after incubation in fluids and agents used peri-operatively to assess antisense oligodeoxynucleotide stability. The gel is stained with 0.2% methylene blue. The first lane shows a bromophenol blue standard which runs at the equivalent of 9 – 10 base pairs. Subsequent lanes show Cx43 AsODN in (A), BSS; (B), rabbit eye vitreous; (C), aqueous; (D), reflex tears; (E), Benoxinate; (F), Pred forte; (G), Fluorescein and (H), Chloramphenicol after incubation for one hour at 37^{o}C. Staining occurs at the same level in all lanes and is well demarcated, indicating that there has been no breakdown of the Cx43 AsODN.

Figure 2: Cx43 expression in uninjured rabbit conjunctiva (A) and cornea (B). Particulate Cx43 labeling (arrows) is visible in the basal layers of the conjunctiva (A). No definitive Cx43 labeling was identified in the sclera and little was identified in the subconjunctival tissue. Cx43 labeling is strong in the basal layers of the corneal epithelium (B) used as a positive control, with some labeling in the stroma. Scale bar = 50 µm.

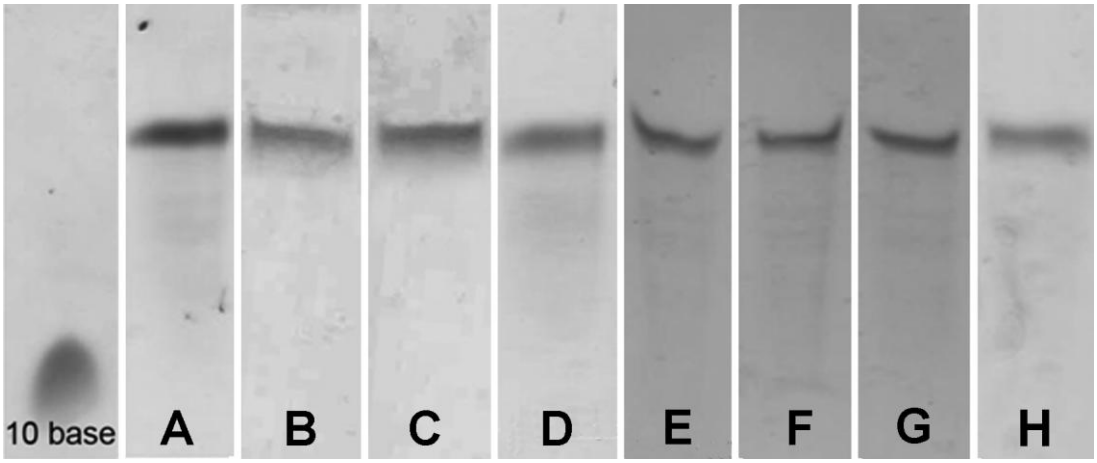
Figure 3: Cx43 expression in rabbit conjunctival and sclera 8 hours following trabeculectomy. Areas adjacent to the cannula opening were examined at higher magnification 500 µm, 1000 µm , and 1500 µm (boxed areas) from the cannula (A). Typical confocal microscope images 500 µm from the cannula site are seen in BSS (B) and Cx43 AsODN treated (C) eyes 8 hours following trabeculectomy. Each image shows the total Cx43 expression within a Z stack projection (volume of 690µm x 230µm x 15µm). Bright spots are gap junctions labeled with a Cx43 specific antibody. The images span the subconjunctival tissue located between the conjunctiva (towards the top of the image) and the sclera (towards the bottom of the image). There is significant up-regulation of Cx43 expression in the BSS treated eye (B) which is attenuated in the Cx43 AsODN treated eye (C). Scale bar = 100 µm. N (Cx43 AsODN, 8 hours) = 3. N (BSS, 8 hours) = 3.

Figure 4: Quantification of the number of Cx43 spots per image at 500 µm, 1000 µm and 1500 µm either side of the cannula site in BSS control and Cx43 AsODN treated eyes at 8 hours after trabeculectomy. A significant increase in Cx43 expression is noted at all regions evaluated in the untreated group compared to the Cx43 AsODN treated group except at -500 µm. The table details the p - values following Student's T-Test analysis. N = 3 (Cx43 AsODN, 8 hours). N=3 (BSS, 8 hours).

Figure 5: Expression of α-SMA in BSS (A) and Cx43 ASODN treated (B) specimens 5 days following trabeculectomy. (A) Increased expression of α-SMA is visible in the subconjunctival tissue (arrows) immediately adjacent to the cannula following BSS treatment. Blood vessel walls (arrowheads) act as positive control labeling within each specimen. (B) Cx43 AsODN treated specimen shows much less α-SMA labeling adjacent to the cannula (auto-fluorescing). Scale bar = 1000 µm. N (BSS, 5 days) = 3. N (Cx43 AsODN, 5 days) = 3.

Figure 6: Montaged hematoxylin and eosin (upper panel) and picrosirius red (lower panel) stained images showing an example of a control trabeculectomy site (A, C) and a Cx43 AsODN treated trabeculectomy site (B, D) 21 days after surgery. (A) The average hematoxylin and eosin grading for the control eye is 7 (out of 8) for the two masked observers. There remains a strong cellular response (purple stained nuclei) and dense packing of collagen fibers (red counter-stain). The scar tissue (densely packed cellular infiltrate) has extended into the lumen of the cannula and the subconjunctival tissue appears firmly adherent to the sclera. (B) The average grading for the Cx43 AsODN treated eye shown is 2.5 for the two masked observers. There is only a minimal cellular response and the collagen is less densely packed. There is minimal scar within the lumen of the cannula although the opening of the cannula is obstructed with scar tissue. There are numerous fenestrations (asterisks) visible within the subconjunctival region and the subconjunctival tissue is loosely adherent to the sclera. (C) Picrosirius red labelling of the control eye demonstrates intense labelling for collagen (red, larger diameter and more tightly packed collagen fibres; yellow, smaller diameter and loosely packed collagen fibres). (D) The collagen is less intensely labelled in the Cx43 AsODN treated eye. Scale bar = 500 µm. N (Cx43 AsODN, 21 days) = 9. N (BSS, 21 days) = 9. N (Pluronic gel, 21 days) = 2.

Figure 7: Persistent anterior chamber (AC) reaction following trabeculectomy. All animals had fibrin with associated flare within the anterior chamber through until at least 3 days post procedure. Cx43 AsODN treated animals resolved the postoperative inflammation significantly sooner (median 5 days) than control animals (median 8 days), Log rank test $p = 0.0262$. N (Cx43 AsODN, 21 days) = 9. N (BSS, 21 days) = 9. N (Pluronic gel, 21 days) = 3.


10 base
A
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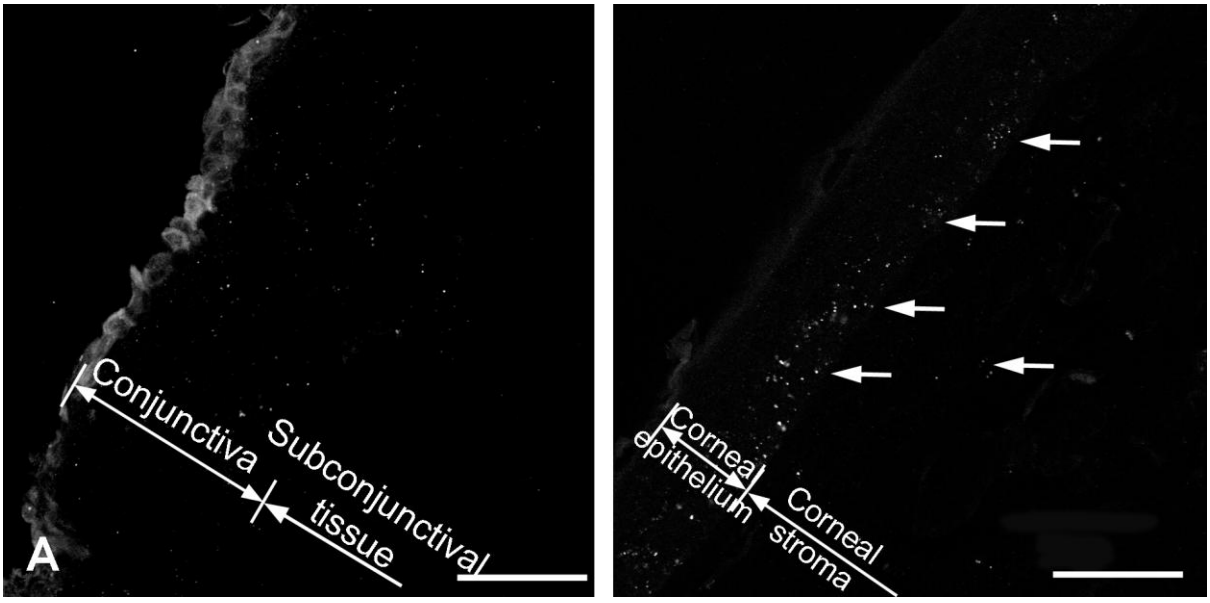
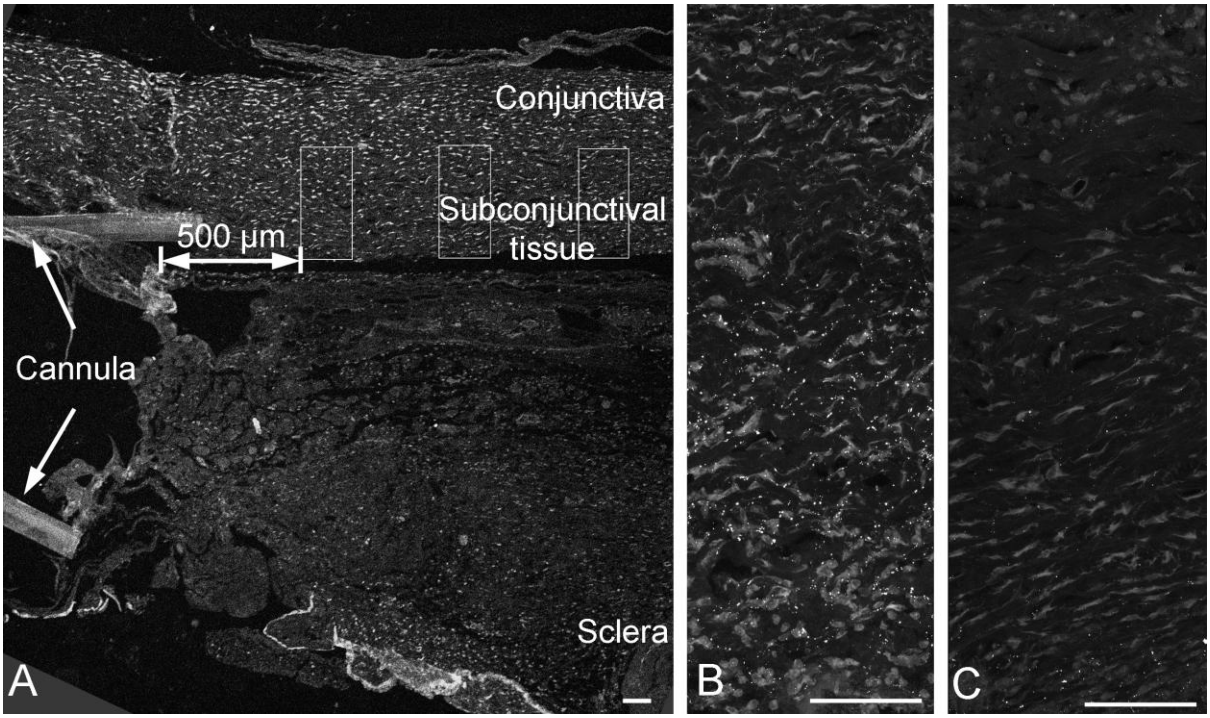
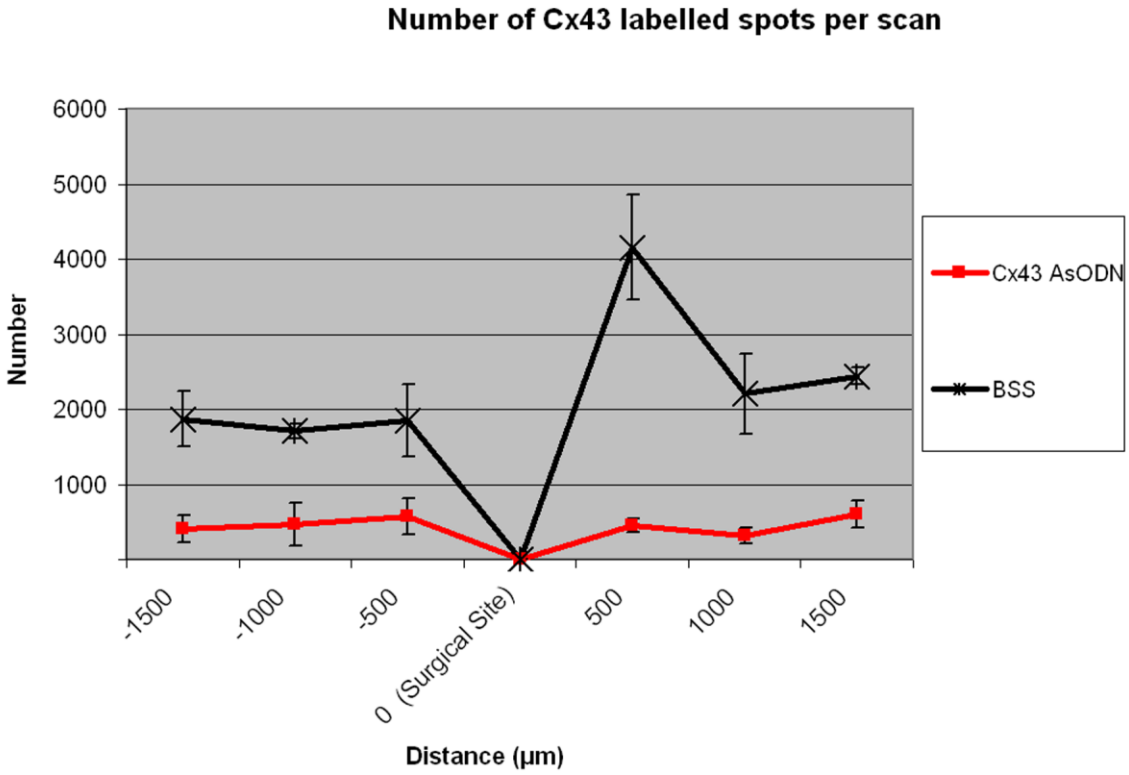
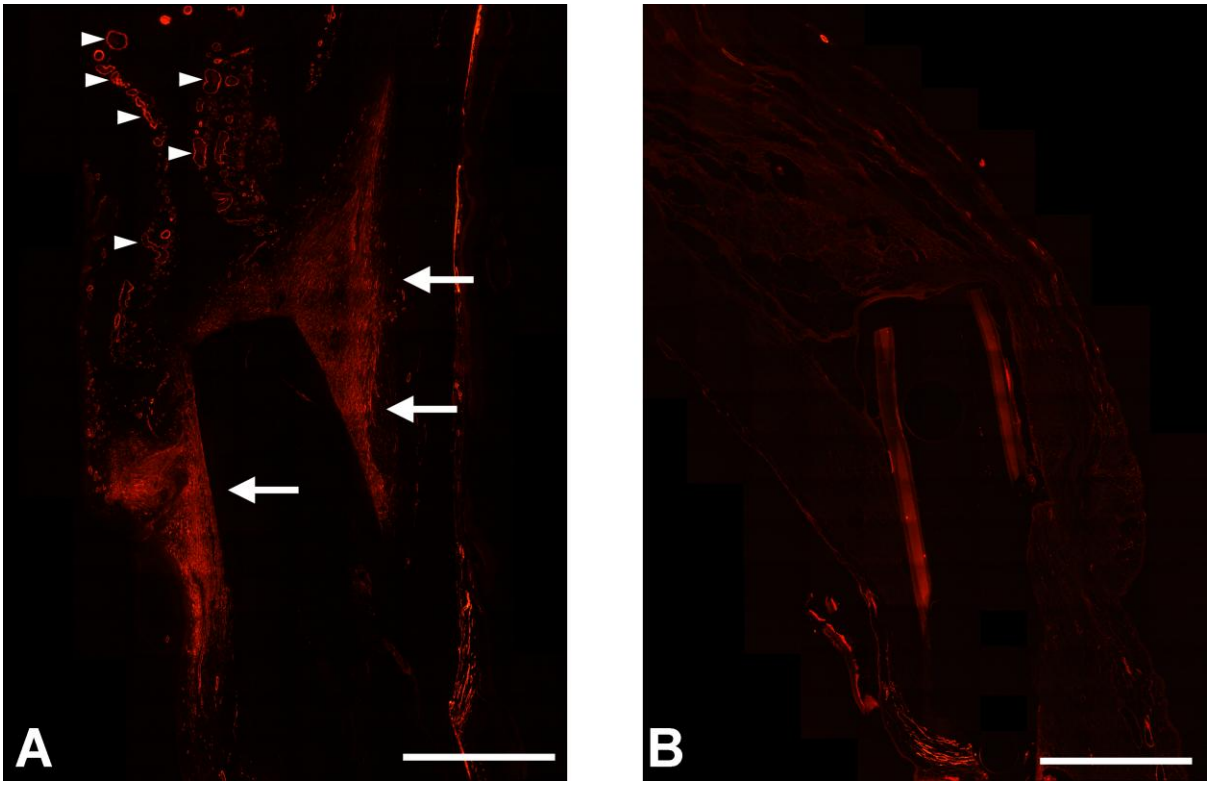
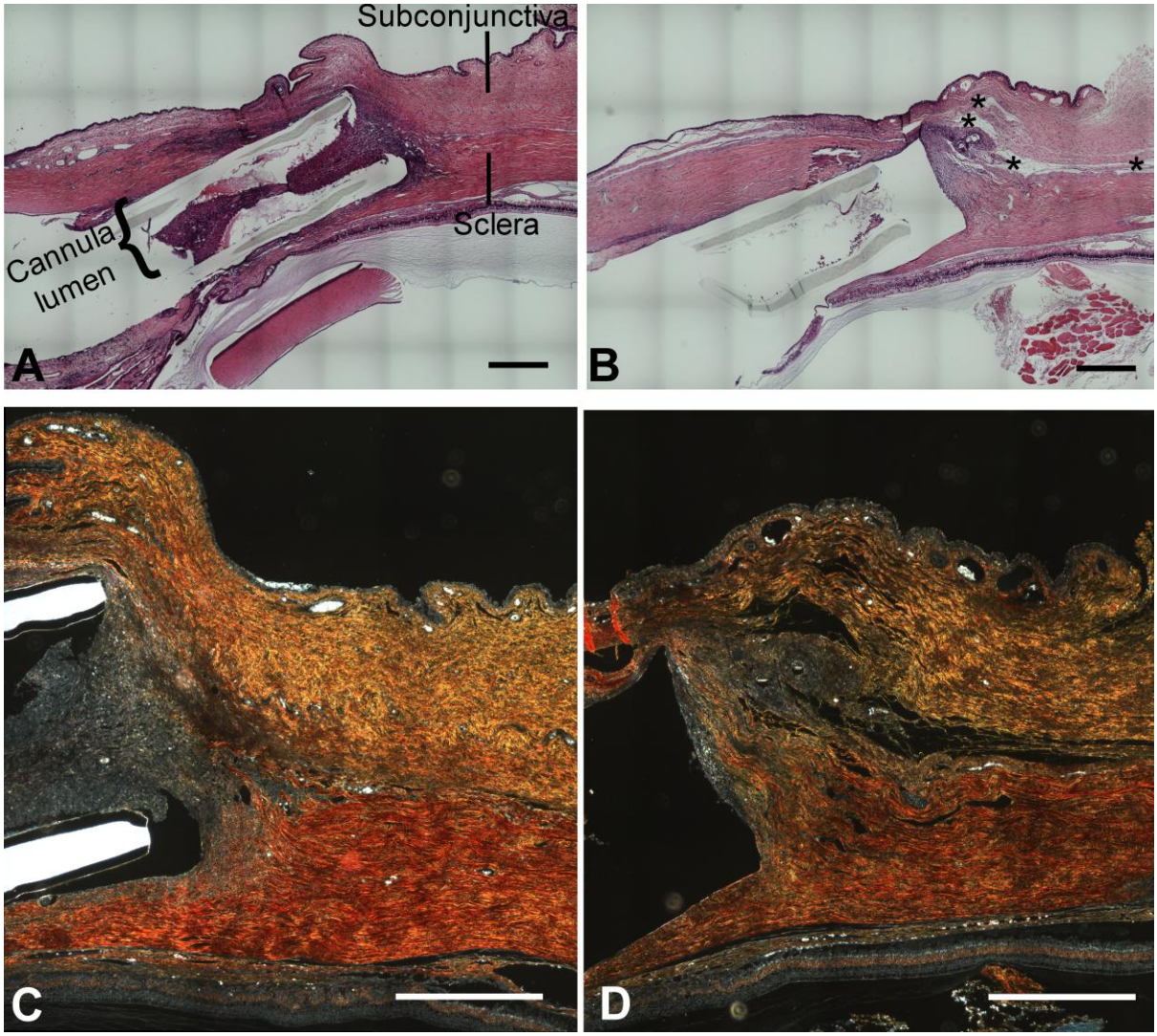

Conjunctiva
Subconjunctival tissue
A
Corneal epithelium
Corneal stroma

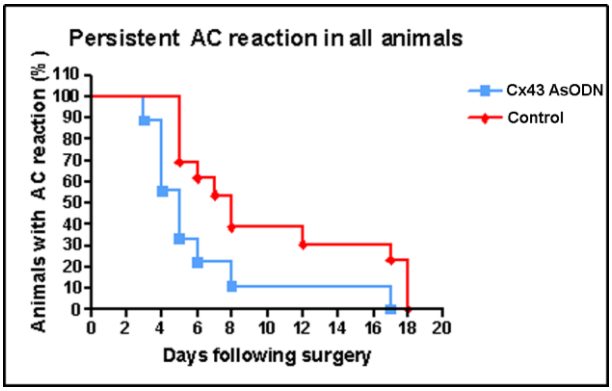

Conjunctiva
Subconjunctival tissue
500 µm
Cannula
Sclera
A
B
C



Site (µm)	-1500	-1000	-500	0	500	1000	1500
p - value	0.022	0.014	0.56		0.006	0.025	0.001


A
B


Subconjunctiva
Sclera
Cannula lumen
A
B
C
D


Persistent AC reaction in all animals
Animals with AC reaction (%)
110
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20
Days following surgery
Cx43 AsODN
Control