In vivo evaluation of intravitreally injected connexin43 mimetic peptide in reducing retinal ganglion cell loss and vessel leak after retinal ischemia

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ABSTRACT SUMMARY
Connexin43 mimetic peptide (Cx3MP) loaded poly(D,L-lactide-co-glycolide) (PLGA) micro- (Mps) and nanoparticles (Nps) were prepared using the double emulsion solvent evaporation method and C12-C12-Cx43MP were synthesised using Boc solid-phase peptide synthesis. Intravitreally injected native Cx43MP and C12-C12-Cx43MP effectively minimized vessel leak, reduced inflammation and protected retinal ganglion cells (RGC) after ischemic injury. Slow Cx43MP release from Nps and Mps was insufficient to have an immediate effect in the treatment of the acute injury, but may provide long-term RGC protection for chronic glaucomatous conditions. Modified peptides, on the other hand, spared 80% of RGC with the effect lasting for at least four weeks. A combination of both immediate and sustained delivery of Cx43MP may ultimately be ideal to achieve optimal RGC survival after an ischemic insult.

INTRODUCTION
Cx43 is the most abundant and important gap junction protein within the body and has been implicated in the pathogenesis of neuronal death after central nervous system injury. At the back of the eye, Cx43 channels play an important role in the maintenance of retinal homeostasis. Up-regulation of retinal Cx43 protein after partial optic nerve injury has been associated with RGC loss and a retinal astrocytic inflammatory response. Therefore, blocking Cx43 mediated vascular leakage using Cx43MP has recently been shown to increase RGC survival to levels near that of uninjured retinas.

The design of Cx43MP has provided a novel tool to quickly and reversibly inhibit the opening of gap junction hemichannels that occurs under certain pathological conditions. Although Cx43MP has shown success in various biological applications, poor tissue permeability and fast enzymatic degradation of the peptides limit their application in a clinical setting. In addition, the prospect of frequent intravitreal injections to treat serious intraocular disorders affecting the choroid and retina has moved researchers to investigate controlled release delivery vehicles such as Mps and Nps.

The aim of this study was to investigate the ability of C12-C12-Cx43MP and Cx43MP containing PLGA Mps and Nps to promote RGC survival in a retinal ischemia-reperfusion rat model by quantifying vessel leak, Cx43 levels and RGC counts.

EXPERIMENTAL METHODS

In vivo ischemia-reperfusion rat model
Rats were anesthetized and the anterior chamber cannulated with a 30G needle attached to an elevated infusion line of sterile 0.9% physiological saline via silicone tubing, with the height of the saline bag calibrated to produce 120 mmHg. This state was maintained for 1 h followed by cannula removal, causing normalization of the IOP and reperfusion of the retina. Intravitreal injection of 2 µl of 20 µM Cx43MP was performed immediately following ischemia-reperfusion.

Quantification of vessel leak, Cx43 and RGC levels
Evans blue solution was injected into the peritoneal space. Animals were rested for 10 min to allow absorption and circulation of the dye through the body prior to euthanasia by asphyxiation. The posterior segment of the eye was carefully removed, the optic nerve and sclera gently detached and the free retina was flattened and mounted onto slides. Besides directly imaging vessel leak, immunohistochemistry was utilized to investigate the effects of ischemia-reperfusion on Cx43 and glial fibrillary acidic protein (GFAP) expression as well as RGC counts. Immunolabelling was visualized with a confocal laser scanning microscope (FV1000, Olympus, Tokyo, Japan) and two fields per quadrant of each retina were imaged giving a total of eight images per retina.

RESULTS AND DISCUSSION
Figure 1 illustrates that uninjured retinas (Fig.1A) did not exhibit any dye leak from the retinal vasculature and vessels were clearly delineated by the dye within them. The dye leak in injured retinas but with no treatment (Fig.1B) was significant compared to uninjured controls (Fig.1A). Cx43MP and C12-C12-Cx43MP (Fig.1C&D) treatment groups showed a reduction in dye leak and blood vessel wall integrity was maintained with branching and segment length similar to uninjured retinas. Qualitative changes in Cx43 and GFAP expression in association with retinal capillaries were observed following retinal ischemia (Fig.2). Cx43 was significantly upregulated and mainly co-localized with GFAP positive astrocytes 8 h after reperfusion (Fig.2B) and continued to increase for up to 28 d (Fig.2C). Intravitreal injection of native Cx43MP resulted in significantly reduced Cx43 upregulation after 8 h (Fig.2D) although this effect was less pronounced after 28 d (Fig.2E). Compared to Cx43 MP treatment where the effects faded by 28 d, C12-C12-Cx43MP containing PLGA Mps and Nps was insufficient to have an immediate effect in the treatment of the acute injury, but may provide long-term RGC protection for chronic glaucomatous conditions. Modified peptides, on the other hand, spared 80% of RGC with the effect lasting for at least four weeks. A combination of both immediate and sustained delivery of Cx43MP may ultimately be ideal to achieve optimal RGC survival after an ischemic insult.
Cx43MP exhibited prolonged results with similar levels of Cx43 and GFAP at both 8 h (Fig.2F) and 28 d (Fig.2G) compared to the uninjured control (Fig.2A). Interesting results were found for the Nps treatment group which displayed increased astrocytosis but limited Cx43 upregulation at 28 d post-injury (Fig.2H). Mps showed similar results at 28 & 90 d (Fig.2I&J) following ischemia-reperfusion with both Cx43 and GFAP upregulated.

Figure 1: Confocal microscope images of flat mounted retinas displaying Evans blue dye leak. (A) uninjured animal; (B) 4h post-reperfusion without treatment; (C) Cx43 MP treatment; (D) C12-C12-Cx43 MP treatment. Scale bar = 100 µm

Figure 2: Confocal microscope images of flat mounted retinas labelled for GFAP (red) and Cx43 (green). (A) uninjured animal; (B) 8 h post-reperfusion without treatment; (C) 28 days post-reperfusion without treatment; (D) 8 h post-reperfusion with Cx43 MP treatment; (E) 28 days post-reperfusion with Cx43 MP treatment; (F) 8 h post-reperfusion with C12-C12-Cx43 MP treatment; (G) 28 days post-reperfusion with C12-C12-Cx43 MP treatment; (H) 28 days post-reperfusion treated with Cx43 MP loaded Nps; (I) 28 days post-reperfusion treated with Cx43 MP loaded Mps; (J) 90 days post-reperfusion treated with Cx43 MP loaded Mps. Scale bar = 50 µm.

Visually, the extent of RGC loss can be observed using Brn3a labeling in representative whole mounts following high pressure induced retinal ischemia and reperfusion (Fig.3). The normal distribution of RGC in flat whole mounts of uninjured retinas is shown in Fig.3A, where a high cell density and clearly outlined retinal vasculature were visible. An example of the degeneration pattern in untreated ischemic retinas is shown in Fig.3B. RGC distribution 28 d after ischemia-reperfusion was significantly reduced with almost complete loss of blood vessel delineation. Eyes treated with unmodified Cx43MP (Fig.3C) showed fewer patches of RGC loss, while the distribution of RGC in the C12-C12-Cx43MP treated group (Fig.3D) was comparable to that of uninjured retinas. Fig.3E and F show the RGC distribution at 28 d in eyes treated with Cx43MP loaded Nps and Mps with some RGC loss apparent, while Fig.3G represents a retina at 90 d after ischemia-reperfusion and receiving a single intravitreal injection of Mps.

CONCLUSION
Intravitreally injected native and modified Cx43MP appeared to effectively minimize vessel leak, reduce upregulation of Cx43 expression and hence rescue RGC after the ischemic injury. C12-C12-Cx43MP showed the most promising results in reducing Cx43 expression down to control levels and sparing 80% of RGC due to increased lipophilicity and vitreous stability. This effect lasted at least four weeks, suggesting that this technique may be a clinically relevant neuroprotective tool in the treatment of glaucomatous optic neuropathy. Cx43 MP loaded Nps and Mps displayed a delayed effect on Cx43 regulation and RGC preservation. A combination of these approaches may ultimately be optimal with the potential to save vision and reduce the treatment burden of retinal diseases by minimizing the injection frequency.

REFERENCES