Intravitreal Tenecteplase (Metalyse) for Acute Management of Retinal Vein Occlusions

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PURPOSE. To determine the ability of an intravitreal injection of tenecteplase (TNK) to penetrate an intraretinal venous thrombus and its effectiveness in thrombolysis in a porcine model of branch retinal vein occlusion (BRVO).

METHODS. Six pigs (group 1) were anesthetized, and a BRVO was induced photothrombotically in the left eye; immediately afterward, fluorescence-conjugated TNK (100 μg) was injected into both eyes, with enucleation at 24 hours. Retinal penetration was assessed on frozen sections by epifluorescence microscopy. A further six pigs (group 2) were anesthetized; BRVO was induced in both eyes, and TNK was injected into the vitreous in the left eye. Both eyes were harvested a week later. The area of the lasered site and an area away from the burn were dissected and processed in epoxy resin and stained for light or transmission electron microscopy. The percentage blockage, clot volume, cytostructure, and extent of thrombolysis by TNK were assessed.

RESULTS. TNK penetrated the veins in both eyes of group 1 pigs, with more intense staining in the eyes with the occlusion. In group 2 eyes, thrombolysis was significant in the eyes injected with TNK (P = 0.03); blockage was seen in all six untreated eyes and one treated eye. Clot volume was significantly higher in untreated eyes (P = 0.028). Percentage blockage varied from 8.5% to 83.9%. Damage by TNK to the neural retina was not seen. There was no significant difference in cytostructure between treated and untreated eyes (P = 0.357).

CONCLUSIONS. TNK was able to penetrate the retinal veins with and without an occlusion and effect lysis of BRVO, and did not cause damage to the retinal tissue. Intravitreal TNK may be useful as an acute treatment for RVOs of recent onset.

Keywords: TNK, BRVO, vitreoretinal surgery

Retinal vein occlusions (RVO) remain a common cause of unilateral vision loss worldwide. They are the second most common cause of retinal vascular blindness after diabetic retinopathy, affecting approximately 16 million people worldwide.1–3 Approximately 1% of individuals younger than 60 years are affected, with the prevalence increasing to 5% in those over 80 years.2

Retinal vein occlusions may affect either a branch vein or the central retinal vein. Branch retinal vein occlusion (BRVO) is more common and accounts for 80% of all vein occlusions.2,4 Without treatment, it can lead to a sustained loss of vision, with a reported final mean visual acuity of 20/70 and 23% of patients having a visual acuity ≤ 20/200.5,6

The vision loss in acute BRVO is predominantly due to macular edema.7 Macular edema and retinal neovascularization are sequelae of the obstruction to venous outflow that occurs in this condition and have been the focus of most of the therapies that have been trialed for this condition to date.

Treatments that potentially directly intervene in the causative pathogenesis have been attempted, but as of yet none have progressed to the stage at which a randomized controlled trial has been performed. BRVOs always occur at an anatomically susceptible spot where an artery and vein cross and the common adventitial sheath binds these together. It is thought that the thickened and rigid arteriosclerotic arterial wall compresses the vein, resulting in turbulence in blood flow and endothelial damage leading to the formation of a local thrombus.8–10

The evidence that a thrombus is the cause of the obstruction to the venous outflow in BRVO is much stronger than in central retinal vein occlusion (CRVO), where both the cause and site of the venous obstruction still remain controversial.11–13 The largest histopathological study of BRVOs showed evidence of a fresh or recanalized thrombus within the retinal vein at the site of the obstruction in all nine cases examined.9 This finding has led to attempts to both relieve the localized obstruction by releasing the pressure on the retinal vein from the adjacent artery, via incision of the common adventitial sheath, and to directly lyse the intravascular thrombus with thrombolytic agents.

Vitrectomy surgery with decompression of the arteriovenous crossing has been attempted, with some reports suggesting a benefit and others finding no difference from vitrectomy alone.14–20 Direct attempts to lyse the presumed thrombus causing the obstruction to venous outflow have also been attempted in both CRVO and BRVO.21–26 These have included injections of tissue plasminogen activator (tPA) both into the vitreous and also directly into a branch vein using a microinjector system. The results from these studies have been...
inconclusive, and intraocular thrombolytics for RVOs are not widely used.

Part of the problem with attempting to lyse the intravascular thrombus in RVOs has been with the agent chosen. Tissue plasminogen activator does not penetrate the retina well and also requires a prolonged clot contact time to be effective. We have previously investigated the third-generation thrombolytic tenecteplase (TNK) as a treatment for submacular hemorrhage. TNK offers significant benefits for intraocular use compared to tPA in that it is less toxic, requires a much shorter clot contact time to be effective, and has been shown to freely penetrate the retina.

In this study we report results on the ability of an intravitreal injection of TNK to penetrate an intraretinal venous thrombus and also to effect a thrombolyis in a porcine model of BRVO.

**Materials and Methods**

All animal procedures were approved by the Animal Ethics Committee of the University of Western Australia and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the policies in the Guide to the Care and Use of Laboratory Animals issued by the National Institutes of Health. A total of 12 pigs were used, 6 to ascertain if TNK can penetrate retinal veins with and without a thrombus, and 6 to determine the thrombolytic properties of and retinal damage by TNK in RVO.

**Surgery and Tissue Processing**

Six pigs (group 1) (aged 18 weeks, body weight ~ 60 kg) were anesthetized and sedated with an intramuscular injection using a combination of Zoletil 100 (tiletamine hypochloride-zolazepam hydrochloride, 4.4 mg/kg; Virbac Australia Pty Ltd., Milperra, New South Wales, Australia) and Illium Xylazine-100 (xylazine hydrochloride, 2.2 mg/kg; Troy Laboratories Pty Ltd., Glendenning, New South Wales, Australia). They were then intubated, ventilated, and maintained on halothane, oxygen, and nitrous oxide. Pupillary dilation was achieved with tropicamide 1% and phenylephrine hydrochloride 2.5%.

Baseline assessment in all eyes included binocular indirect ophthalmoscopy. After intravenous injection via an ear vein of 10 mg/kg Rose Bengal dye (Sigma Aldrich, North Ryde, New South Wales, Australia), which is a dye with peak absorption of light close to the wavelength of the argon laser and allows an intravascular thrombus to be created with minimal damage to the vessel wall with use of appropriate laser powers. A photothrombotic BRVO was attempted in the vein adjacent to the optic disc in the left eye of each pig using an argon green laser (514 nm wavelength) as described previously. In order to avoid washing away labeled protein/TNK, sections were analyzed and photographed without any other procedures having been performed. Measures were taken to minimize exposure to light; electrical lighting was avoided, and frozen blocks and sections were stored in lightproof containers. The distribution of the labeled protein was visualized under an epifluorescent microscope (Nikon Eclipse E-800; Nikon, Tokyo, Japan) equipped with fluorescence-relevant detection filter (excitation/emission maxima 495/519 nm). To ensure that the fluorescence distribution and intensity were related only to the amount of TNK present, all the observations and photographs were obtained at a preset setting on the microscope, which was used for all the sections in this analysis.

A further six pigs (group 2) (aged 4 weeks, body weight ~ 10 kg) were anesthetized, sedated, intubated, and ventilated and had pupils dilated as described above. Baseline assessment in all eyes with binocular indirect ophthalmoscopy was performed. A photothrombotic BRVO was attempted in both eyes as described above. One week after creation of the occlusion, eyes were reexamined by ophthalmoscopy; and intravitreal injection of TNK 100 μg/0.1 mL saline via the pars plana 2 to 3 mm posterior to the limbus using a 30-gauge needle was performed in the left eye. The fellow eye (right) had no injection. Chloramphenical ointment was again applied to the eyes after surgery. After 24 hours the eye cup was photographed, and full-thickness sections from the left eyes, from the same vein of the right eyes with no occlusion, and from the control eye were dissected and used for assessing the effects of TNK on the retina.

Both the TNK-treated and nontreated enucleated eyes had a small slit made just below the limbus and were immediately fixed in 2.5% gluteraldehyde in 0.1 M phosphate buffer. After 24 hours the eye cup was photographed, and full-thickness pieces of retina and choroid 2 mm² were dissected from the relevant area containing the laser burns. Farther away from the laser burn, outside the area of the BRVO, additional sections were dissected for assessing the effects of TNK on the retina. One treated eye was discarded due to a retinal detachment with the vitreous forming an opaque solid mass. The specimens were postfixed in osmium tetroxide, dehydrated in a graded series of ethanol, and thereafter infiltrated and embedded in epoxy resin.

Two-micrometer spaced (every 20 μm) semithin serial sections were cut, stained with toluidine blue for light microscopy (LM), and photographed at oil emersion ×40 and ×60 magnification. Sections from the area away from the laser...
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In the TNK-treated eyes, only one out of the five had a partial occlusion with narrowing of the lumen. The other four treated eyes showed no evidence of an occlusion (Fig. 5) \((P = 0.03)\). ImageJ analysis and computation presented volume of clot of the untreated and treated eyes varying between 0.0000426 mm\(^3\) and 0.0204 mm\(^3\), with volume size significantly greater in the untreated eye \((P = 0.028)\). The percentage blockage of the segment of vein occupied by the thrombus in untreated and TNK-treated eyes ranged between 85% and 83.9% (Table). The shortest length of vein occupied by the thrombus was 0.4 mm and the longest 3.98 mm; both of these were seen in the untreated eyes. Histological examination by LM of the thrombus in the untreated eyes revealed stagnant red blood cells, some lysed, fragmented, and/or clumped together and interspersed by polymorphonucleocytes. The vein wall was intact in all eyes, untreated and TNK treated, but was surrounded by macrophages, some of which were laden with pigment granules.

There was no evidence of damage by TNK to the retina (Fig. 4). No difference was discernible under LM between TNK-treated and untreated eyes (Fig. 4). Retinal pigment epithelial cell nuclei were rounded and healthy. Pigment granules appeared typical, dispersed within the cell most toward the apices of the cells. Rod outer segments were mildly disorganized, and some pyknotic cells and swelling of the inner retinal layer and in the ganglion cell layer were also seen in both untreated and TNK-treated eyes. TEM in both eyes showed (Fig. 5) no damaged cells in the retinal pigment epithelial layer. These cells appeared to be active, with apical microvilli engulfing rod outer segments. An occasional pyknotic nuclei was seen in the outer nuclear layer. A few cells showed vacuolar damage and mild swelling in the inner retina. There was also some swelling in the ganglion cells; however, damaged cells were rare. The cells were endowed with a large number of intracellular organelles including mitochondria, some of which showed mild swelling in both untreated and treated eyes. Inflammatory cells were not seen in any of the eyes examined. TEM confirmed LM findings with no significant difference in necrosis between untreated and TNK-treated eyes \((P = 0.357)\).

**DISCUSSION**

Since the publication of the Branch Vein Occlusion Study (BVOs) 25 years ago, grid laser photocoagulation has been the standard of care for BRVO-associated macular edema. In this study, treatment of macular edema following BRVO with grid laser resulted in an average gain of 1.1 lines of vision compared to that in control eyes at the 3-year primary end point. In the last 5 years, several new treatments for macular edema secondary to BRVO have been evaluated in randomized clinical trials. These included intravitreal triamcinolone injections, intravitreal dexamethasone implants, and inhibitor of vascular endothelial growth factor (VEGF) agents. Of these, anti-VEGF agents have been the most promising. In the Ranibizumab for the Treatment of Macular Edema following Branch Retinal Vein Occlusion (BRAVO) study, rapid and sustained visual improvement was seen in patients who received monthly 0.3 mg and 0.5 mg ranibizumab compared with sham injections. At the 6-month primary end point of the study, the mean gain from baseline best-corrected visual acuity (BCVA) letter score was 16.6 and 18.3 Early Treatment Diabetic Retinopathy Study (ETDRS) letters in the 0.3 and 0.5 mg ranibizumab cohorts, respectively, compared with 7.3 letters in the sham group. After 6 months, treatment with ranibizumab on an as-needed basis in all patient groups showed maintenance of the visual gain that was achieved in the ranibizumab groups at the 1-year end point.
Figure 1. Photothrombotic branch retinal vein occlusion. (A) Macroscopic view of a normal pig eye indicating the position of the attempted laser photocoagulation (marked). (B) Transmission electron micrograph of an untreated pig eye in group 2, in which a branch retinal vein occlusion was created, showing retinal vein lined by normal-looking endothelial cells (E) with a platelet adjacent to it and lumen occupied by a thrombus containing stagnant red blood cells (RBC), a polymorphonucleocyte (blue arrow) with invaginated nuclei, and phagocytizing cell debris and fibrin-like material (red arrow). Scale bar: 1.4 μm. (C) Transmission electron micrograph of untreated pig eye in group 2, in which a branch retinal vein occlusion was created, showing an area of the thrombus composed of packed stagnant red blood cells (asterisks) and several others that are lysed and fragmented. Scale bar: 1.4 μm. (D) Macroscopic view of the eye cup of an untreated eye showing laser burn site (marked) and attenuation of flow of blood (arrowheads) toward the optic disc (OD). (E) Macroscopic image of the eye cup of a TNK-treated eye showing laser burn site (marked) and flow of blood to the optic disc (OD).

Figure 2. Retinal penetration after intravitreal injection of fluorescent-labeled TNK. (A) Epifluorescence micrographs of a frozen section of a normal abattoir pig retina from group 1 eyes taken close to the optic disc in the region of the vein. Faint red autofluorescence of blood cells (arrow) in the retinal vein with all other neural layers devoid of fluorescence is seen. Scale bar: 70 μm. (B) Epifluorescence micrographs of a frozen section of a retina in group 1 pig eye without a created branch vein occlusion and with injected intravitreal fluorescent-labeled TNK, showing staining within the retinal vein (RV) and stronger staining in the vein wall, inner limiting membrane, outer nuclear layer, and rod outer segments. Scale bar: 70 μm. (C) Epifluorescence micrographs of a frozen section of a retina in a group 1 pig eye with a branch vein occlusion created and with injected intravitreal fluorescent-labeled TNK, showing strong staining within the retinal vein (RV), in the vein wall, and inner limiting membrane. Scale bar: 70 μm.
The BVOS group also demonstrated the effectiveness of scatter laser in reducing the development of retinal neovascularization, which is another late sequela of BRVO.86–88

While these results are impressive and represent a major advance in our ability to improve the visual results in this condition, these treatments are all aimed at the sequelae of the condition and make no attempt to directly modify the disease process. BRVOs occur at an arteriovenous crossing, with strong evidence that an intravenous thrombus is the penultimate cause of the occlusion to venous outflow and that there is further active propagation of the thrombus downstream.88–90 It is this occlusion that is responsible for the delay in retinal blood flow through the occluded segment and the subsequent VEGF upregulation and elevation in hydrostatic pressure that produce the retinal changes seen clinically.91–93 Results from the BRAVO and subsequent extension studies have shown that the VEGF upregulation can be controlled with intraocular injections of ranibizumab with good initial effects on visual acuity; however, injections in many patients need to be continued for years.94–96 The other potential component of the macular edema is the elevation of the venous hydrostatic pressure in the occluded segment. This is resolved only once either the clot is recanalized or hemodynamically significant collateral circulation occurs. Collateral vessels have been noted to take between 2 and 18 months to appear after BRVO and may take additional time after this to develop to the stage where they have any significant effect on the elevated venous hydrostatic pressure.97 A treatment aimed directly at relieving the obstruction to venous outflow may potentially play a role additional to current conventional anti-VEGF therapies.

Thrombus formation is a dynamic process, and sheer forces, flow turbulence, and platelets in the circulation all can greatly influence the architecture of the thrombus.98 In this study there was some variability in the volume size of the thrombus and the distance of vein occupied by it. This variability may be attributed to propagation by further recruitment of clot elements in the larger thrombi or, in the smaller thrombi, the initiation of recanalization and/or clot retraction to renew blood flow.92

The porcine retina is holangiotic and has a vascular supply very similar to that of the human retina. In this study we investigated the ability of a 100 μg intravitreal injection of TNK to penetrate the retina and bind to and lyse an experimentally created intravenous thrombus in a porcine animal model. TNK was shown to penetrate all layers of the retina in the eyes in group 1, with labeled TNK seen in the lumen of the retinal vein in eyes both with and without a BRVO. We have previously also demonstrated that TNK has the ability to penetrate all the layers of the retina 6 and 24 hours after intravitreal injection.99 In the eyes with a BRVO, the labeled TNK is seen bound within the clot, showing that this agent has the ability to penetrate the retina and bind to an intravenous thrombus within 24 hours.

In the eyes in group 2, an intravitreal injection of TNK successfully lysed an intravenous thrombus in 80% of the eyes by 2 weeks after the injection compared to the control group (P = 0.03); all eyes with a BRVO and no intravitreal TNK showed persistence of the thrombus at the same time point and significantly greater clot volume size (P = 0.028).

We have previously investigated the safety of intravitreal TNK to the inner and outer retina, its ability to penetrate the retina, and its ability to effectively lyse subretinal hematomas in animals and humans.99–103 Tissue plasminogen activator has been extensively used in humans in an attempt to lyse clots and fibrin collections; however, significant questions remain concerning its effectiveness, potential toxicity, and ability to penetrate the retina. It was originally developed for use in myocardial infarction; however, because of its short half-life, the treatment involved a

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**Figure 3.** Effect of intravitreal injection of TNK on photothrombotic branch retinal vein occlusion. (A) Light micrograph of a group 2 untreated pig retina in which BRVO was attempted, showing the lumen of the vein occupied almost completely by a thrombus packed with red blood cells clumped together and interspersed by inflammatory cells. The retinal vein is surrounded by macrophages (arrow). Scale bar: 11 μm. (B) Light micrograph of a group 2 pig retina in which BRVO was attempted and treated with TNK, showing the lumen of the vein containing a few red blood cells occupying a small area of the lumen. Pigment-laden macrophages (arrows) surrounding the vein are seen. Scale bar: 11 μm.
bolus injection followed by a two-step 90-minute infusion. The effectiveness of its use intravitreally has also been questioned in the treatment of submacular hemorrhage (SMH). It binds very strongly to the ILM of the retina, which limits its ability to act either within the retina on an intravascular thrombus or underneath on a SMH. In a study on rabbit eyes, labeled tPA was found layered on the retinal surface after an intravitreal injection, with no penetration into the retina. An additional study of tPA retinal penetration by Mahmoud et al. in porcine eyes showed conflicting results. The investigators used an occlusion/reperfusion model of RVO and found some evidence of tPA penetration using an antibody to tPA by immunoperoxidase histochemistry. Owing to variable background staining in their negative controls in the immunoperoxidase staining, they performed indirect immunofluorescence staining of the tPA with conflicting results; no fluorescence staining was seen within the retinal veins either with or without an occlusion. Both methods showed intense staining at the level of the ILM, highlighting the barrier effect that this layer has to retinal penetration by tPA. It remains unanswered if indeed tPA penetrated the lumen of the retinal veins in that study, considering the variability in the background staining in the control samples, the absence of staining in the lumen of the veins by indirect immunofluorescence histochemistry, and no

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Figure 4. Effect of intravitreal injection of TNK on retinal histology. (A) Light micrograph of a group 2 untreated pig retina, section away from the laser burn. The retina looks normal. Retinal pigment epithelium (RPE) appears normal and healthy with rounded nuclei, mildly disorganized rod outer segments (OS), outer nuclear layer (ONL) containing a few pyknotic cells, and mild swelling of cells in the inner nuclear layer (INL) and ganglion cell layer (GCL). Scale bar: 17 μm. (B) Light micrograph of a group 2 pig retina treated with intravitreal injection of TNK, section away from the laser burn showing a normal healthy-looking retina similar to the untreated pig retina, with retinal pigment epithelium (RPE) appearing normal and healthy with rounded nuclei, mildly disorganized rod outer segments (OS), outer nuclear layer (ONL) containing a few pyknotic cells, and mildly swollen cells in the inner nuclear layer (INL) and ganglion cell layer (GCL). Scale bar: 17 μm.
Figure 5. Effect of intravitreal injection of TNK on retinal ultrastructure. Transmission electron micrograph from group 2 untreated and treated pig retina, section away from the laser burn showing similar ultrastructure. (A) Untreated, (E) treated: ganglion cell layer including a ganglion cell with nucleus (N) and mitochondria mildly swollen in both eyes. (B) Untreated, (F) treated: inner nuclear layer cells with nuclei (N) showing swelling in some cells in both eyes. (C) Untreated, (G) treated: outer nuclear layer with regularly arranged nuclei (N) in both eyes. (D) Untreated, (H) treated: Retinal pigment epithelium cells appear normal and healthy in both, with pigment granules in the apical region of the cell and microvilli (arrows) engulfing rod outer segments (asterisks) in both eyes. Scale bar: 2 μm.
A clinical trial to answer these questions, and also address the need for other supplemental medications to prevent reoccurrence of the thrombus in the acute phase, will be required and is currently being planned.

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