MCKENZIE, B., KAY, G., MATTHEWS, K.H., KNOTT, R.M. and CAIRNS, D. 2015. The hen's egg chorioallantoic membrane (HET-CAM) test to predict the ophthalmic irritation potential of a cysteamine-containing gel: quantification using Photoshop® and ImageJ. *International journal of pharmaceutics* [online], 490(1-2), pages 1-8. Available from: https://doi.org/10.1016/j.ijpharm.2015.05.023

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2015

The chorioallantoic membrane (HET-CAM) test to predict the ophthalmic irritation potential of a cysteamine-containing gel.

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Abstract

Nephropathic cystinosis is a rare recessive disease characterised by raised lysosomal levels of cystine in the cells of all organs. It is treated by regular oral administration of the aminothiol, cysteamine. Corneal crystal deposition is one of the most troublesome complications affecting patients and requires the hourly administration of cysteamine eye drops. In an attempt to reduce this frequency and improve the treatment, the preparation and evaluation of cysteamine containing hyaluronate gel is reported. The tests demonstrated that the novel gel formulation is non-irritant to the ocular tissues, in line with saline solution (negative control). In conclusion these results demonstrate that cysteamine can be formulated as an ocular applicable gel formulation.

Keywords: Cystinosis, cysteamine, HET-CAM test, ImageJ, Photoshop®, ophthalmic.

1.0 Introduction

Nephropathic cystinosis is a rare autosomal recessive disease caused by a homozygous mutation in the CTNS gene which codes for cystinosin, a lysosomal membrane transport protein for cystine[1]. As a result, cystine cannot egress the lysosome and instead accumulates and crystallises, ultimately causing organ damage. Cystine crystals accumulate in almost all organs and tissues in the body, although the kidneys and eyes are particularly susceptible[2]. The main clinical parameter for diagnosis and long-term monitoring is extremely high levels of leukocyte cystine[3]. The disease results in death from renal failure by the second decade of life unless cystine levels can be reduced and kept within acceptable limits (< 1 nmol half-cystine/mg protein). The main symptoms of the condition are poor growth, renal Fanconi syndrome, renal glomerular failure and impairment of other tissues and organs. If oral cysteamine treatment is initiated just after birth this may attenuate the rate of renal failure, however, glomerular damage present at the time of diagnosis is irreversible and may result in the need for kidney transplant[4-7].

Although novel prodrug strategies are being researched[8] the main treatment for the disorder remains the oral administration of the aminothiol, cysteamine (figure 1) (as the bitartrate salt, Cystagon™). The molecule lowers intracellular levels of cystine by forming a cysteamine–cysteine mixed disulfide. The disulfide is spatially and structurally similar to the amino acid lysine and can egress the lysosome using the undamaged excretion pathway for lysine[9]. Due to a lack of vasculature in the cornea,

the oral form of cysteamine has no effect on depleting corneal crystals, thus cysteamine must also be applied topically in the form of eye drops[10].

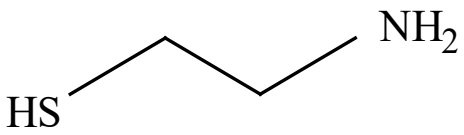

Figure 1. Cysteamine

Corneal crystal deposition is one of the most troublesome complications affecting patients, especially as their prognosis improves and life expectancy increases. Crystals deposit in the cornea slowly from birth through infancy until they become a problem, usually around the age of 6-8 years[11]. Photophobia and, ultimately, blepharospasm affect the quality of life such that any sunlight can be debilitating. The crystals' accumulation over a period of years can cause corneal scars, keratitis and cataracts, as well as band keratopathies to form[12].

The current treatment for the corneal crystals is an ophthalmic solution of cysteamine hydrochloride, which has demonstrated efficacy in depleting corneal crystals following compliant treatment[11,12]. However, the eye drops have to be administered every hour while awake in order to achieve maximum benefit, with a large treatment gap overnight. When inserted, the eye drops cause discomfort, due the low pH of the drops[13]. Despite excellent patient compliance, eye drops are rapidly drained from the ocular surface. In order to improve the bioavailability of ophthalmic delivery

systems, slow the progression of crystal deposition, while also allowing freedom from an 8 to 15-a-day dosing schedule, it is desirable to prolong ocular residence time and encourage site-specific delivery through sustained drug release. Ophthalmic gels may provide a viable alternative to the current eye drop formulation.

An eye gel has been developed and characterised in our laboratories[14]. Part of this process is to elucidate the ophthalmic irritation potential of the eye gel. One model for this is the Draize Rabbit Eye Test, which is very invasive and involves the slaughter of the adult rabbit, and subsequent removal of the rabbit eye[15,16]. The alternative for this test is the hens' eggs test using the chorioallantoic membrane (HET-CAM test), an inexpensive and sensitive assay which uses fertilised hens' eggs[17]. The HET-CAM test is an established test for ophthalmic irritancy, and has shown good correlation to ophthalmic irritation in the vivo situation[15,18-20]. The membrane separates the embryo from the airspace, and is non-innervated, highly vascularised and responds to injury in a similar way to rabbit conjunctiva[16,20]. The HET-CAM test sits between in vivo and in vitro techniques, and is not a licensed procedure.

The ophthalmic gel formulation reported in this paper has the potential to provide an effective treatment for the corneal crystals, due to its bioadhesive and pseudoplastic properties, while relieving the discomfort which is currently experienced by formulating an isotonic gel at pH7.4. The ophthalmic gel may also allow fewer doses to be administered, lessening the dosage burden through prolonged contact with the cornea. Sodium hyaluronate is a natural, high molecular weight polymer which is expressed in human vitreous humour[21], and therefore provides an inherent

biocompatibility with the eye[22]. It also promotes wound healing[23], and has been used commercially in artificial tears preparations for over 30 years[23] and has an established safety record[21].

Current understanding of ocular pharmacokinetics involves mixing of the eye drops with lachrymal fluid, produced at a rate of 0.5-2.2 μL/min[24,25], resulting in a short contact time with ocular tissue. Subsequent drainage towards the nasolachrymal duct during blinking results in extensive elimination of the applied solution and contact times varying from 1-2 minutes[26,27] to 5 minutes[28-30] have been reported. The rapid drainage rate is due to the tendency of the eye to maintain its residence volume at ~10 μL, and consequently, the overall absorption and bioavailability of a topically applied drug is typically less than 5%[31,32]. An ophthalmic gel may overcome these physical barriers and improve bioavailability through improved contact time with the corneal surface[33].

The sodium hyaluronate hydrogel is both transparent and bioadhesive and these attributes are highly desirable for topical ophthalmic application[34]. Non-Newtonian pseudoplastic or 'shear thinning' rheology facilitates the process of blinking by dramatic reductions in apparent viscosity as a function of the high external shear stresses applied by the leading-edge and inside surface of the eye-lid[35]. These high shear stresses are associated with equivalent shear rates of 0 s^{-1} at rest to 10,000 - 40,000 s^{-1} when blinking[36]. Pseudoplastic fluids therefore offer significantly less resistance to blinking than Newtonian liquids of equivalent viscosity. High apparent viscosities under zero external stresses result in longer contact times on the surface of

the eye[37]. In addition to these physico-chemical properties, the gel is isotonic due to the inclusion of SMPB buffer as an excipient[38], and sits at physiological pH7.4, minimising the potential for irritation. For topical ophthalmic formulations of cysteamine, these properties should result in less frequent application and better patient compliance.

The aim of this work is to elucidate the effect of the eye gel and the current ophthalmic solution of cysteamine on the blood vessels of the egg's chorioallantoic membrane (HET CAM test), which demonstrates good correlation with the eye and is an established ophthalmic model[16]. The outcomes of this study could provide rationale for the usefulness of the gel in the treatment of the ophthalmic complications of cystinosis.

2.0 Materials and methods

2.1 Materials

Cysteamine hydrochloride and benzalkonium chloride were purchased from Sigma (Dorset, UK). Sodium hyaluronate was obtained from Aromantic Ltd (Moray UK). Water for injection (WFI) was made by autoclaving distilled water. Saline solution was made using sodium chloride obtained from Fisher, dissolved in WFI. Fertilised hens' eggs were obtained from a local breeder. All procedures conformed to current

national guidelines: eggs were used within 10 days of incubation. All other chemicals were of general laboratory grade.

2.2 Preparation of gels

The medicated gels at pH 7.4 were made by the addition of sodium hyaluronate to WFI, which was allowed to fully hydrate overnight at 4˚C. After stirring for 5 minutes, the pH of the gel solution was then adjusted to 7.4, by addition of 2M hydrochloric acid. Sorensen's Modified Phosphate Buffer (SMPB) was added, along with cysteamine hydrochloride. The pH was maintained at 7.4. Benzalkonium chloride was added as a preservative. The final weight of the gel (20g) was made up with WFI. All gels were allowed to rest at 4˚C for 24h until further testing commenced.

The unmedicated gels were manufactured as above but without cysteamine. The gels with a modified pH were manufactured with 2M NaOH or 2M hydrochloric acid to reach the desired pH. The medicated solutions were made as per the current formulation, i.e. cysteamine hydrochloride in a saline solution, with benzalkonium chloride as a preservative[39,40].

2.3 HET-CAM test: method

The fertilised hens' eggs were obtained on day zero. They were either Dorking-Wyandotte crosses or Dorking-Rhode Island Red crosses. The eggs were marked with crosses, numbers and dates and incubated at 37±0.5˚C and 40%±5% humidity for 9 days[41]. The eggs were incubated in the horizontal position to ensure the correct positioning of the embryo (away from the CAM). They were manually rotated 180˚at least three times a day for the duration of the test, to ensure correct development and viability of the embryo. The exact time that they were placed in the incubator was marked as T_0. On day 9, the eggs were candled to ensure fertility, and the shell was marked on the line of the airspace. When the eggs had been incubated for 10 full days, the experiment began. Each egg was removed individually from the incubator, and placed in an egg holder with the larger end upwards. The shell was cut just above the marked line of the chorioallantoic membrane using a Dremel® Multitool (Illinois, USA), with rotating cutting blade attachment. Once this section of shell had been removed, the inner membrane directly in contact with the CAM was moistened with 2ml of 0.9% saline solution, added with a pippette. The inner membrane was then carefully removed using forceps, without causing injury to the blood vessels, to reveal the chorioallantoic membrane below. The same volume of test solution was then added directly onto the CAM using a pippette, and a timer started. Any lysis, haemorrhaging and/or coagulation at different times over a 5-minute period after application of the test solution will be documented, and any effect is noted and compared with controls: saline (negative) and sodium hydroxide (positive) solutions. Photographs were taken to record qualitative data. Once this 5-minute period was over, the membrane was disrupted, the remains of the egg placed into a sealed bag,

and the bag placed into the freezer for subsequent incineration. Each egg was treated in this way until all of the eggs had been tested and destroyed. Each experimental protocol was replicated 5 times .

A semi-qualitative analysis was performed using the photographs, where the severity of any haemorrhage was graded on a scale from 0 (no reaction) to 3 (strong reaction) using the method developed by Gupta et al[20]. The photographs, obtained using a Canon Powershot digital camera were subsequently analysed using Adobe® Photoshop®[42] and ImageJ[43] (available as freeware from http://rsb.info.nih.gov/ij/) to quantify the vascular damage, allowing a more detailed and robust analysis of the gels to be made[16, 44-46].

2.4 HET-CAM test: quantification using Photoshop® and ImageJ

The photos of the chorioallantoic membranes were loaded individually onto Photoshop®, cropped for size, and the image converted to greyscale. The resulting files were then saved individually as TIFF files with no layers[44].

To quantify the extent of any haemorrhaging, the TIFF files formed using Photoshop® were loaded into ImageJ. To analyse the greyscale values of the pixels over a standardised length of membrane, the menu option ‘Lines’ was selected, and a straight line drawn across the image. The profile of the average grey pixel value along the

drawn line was plotted, and the length of the line adjusted to a uniform length. The menu option 'List' was selected and the values copied into Microsoft® Excel® (Washington, USA). This process was repeated for all images, and the average pixel grey value plotted against distance[44].

3.0 Results and discussion

Application of 2ml 0.9% saline solution to the healthy membranes produced no visual response over the five-minute period (figure 2). In contrast, 1M NaOH produced a severe, instant haemorrhage, which increased over five minutes (figure 3), grading this solution as a severe irritant[47].

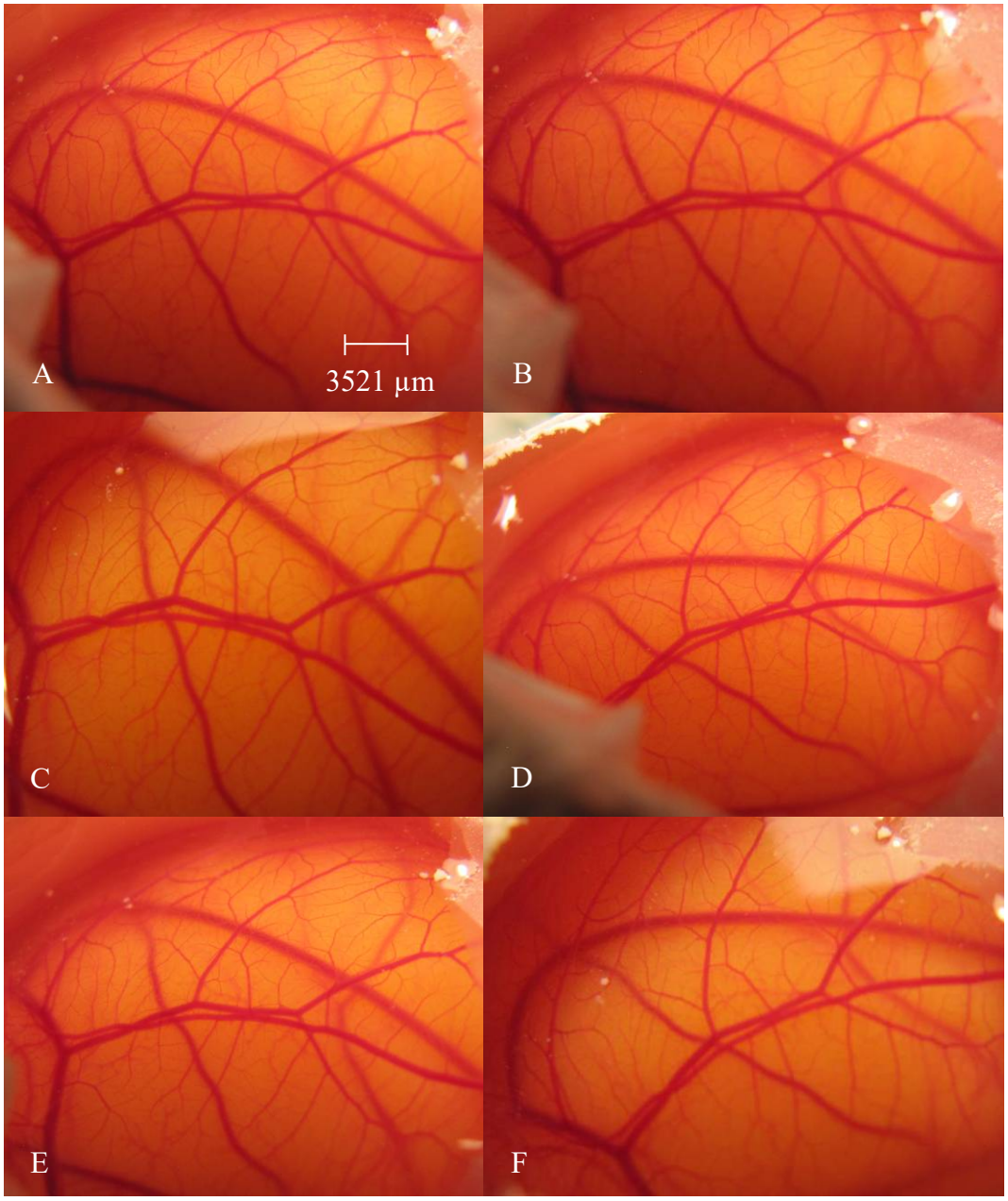

Figure 2. Sequence of photographs illustrating the effect of 2ml 0.9% saline solution (negative control) on the membrane over a 5-minute period. A. Healthy membrane at T_0, B. Membrane at $T_{0.5minutes}$, C. Membrane at $T_{1minute}$, D. Membrane at $T_{2minutes}$, E. Membrane at $T_{4minutes}$, F. Membrane at $T_{5minutes}$.

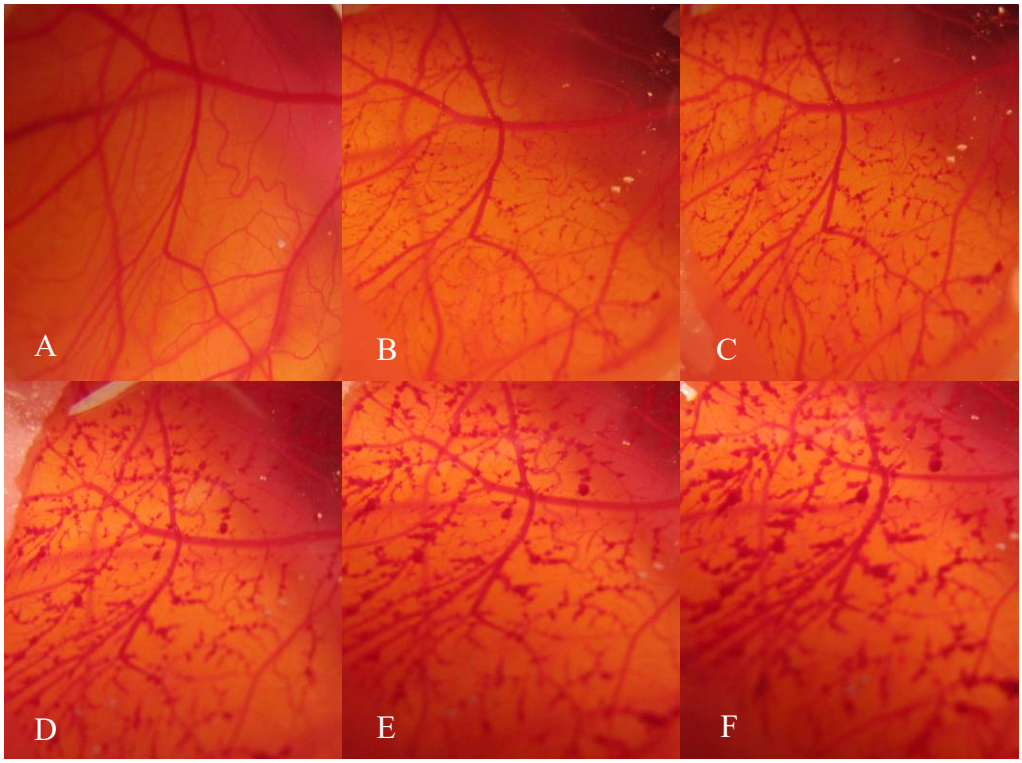

Figure 3. Sequence of photographs illustrating the effect of 2ml 1M NaOH (positive control) on the membrane over a 5-minute period. A. Healthy membrane at T_0, B. Membrane at $T_{0.5minutes}$, C. Membrane at $T_{1minute}$, D. Membrane at $T_{2minutes}$, E. Membrane at $T_{4minutes}$, F. Membrane at $T_{5minutes}$.

The unmedicated gel at pH7.8 produced a faint reddening of the membrane gradually over the time period (Figure 4). The mildly alkaline nature of the gel (pH7.8) is on the upper threshold of ocular tolerance limits (pH6.6-7.8), beyond this range patients can experience stinging or discomfort[48-51].

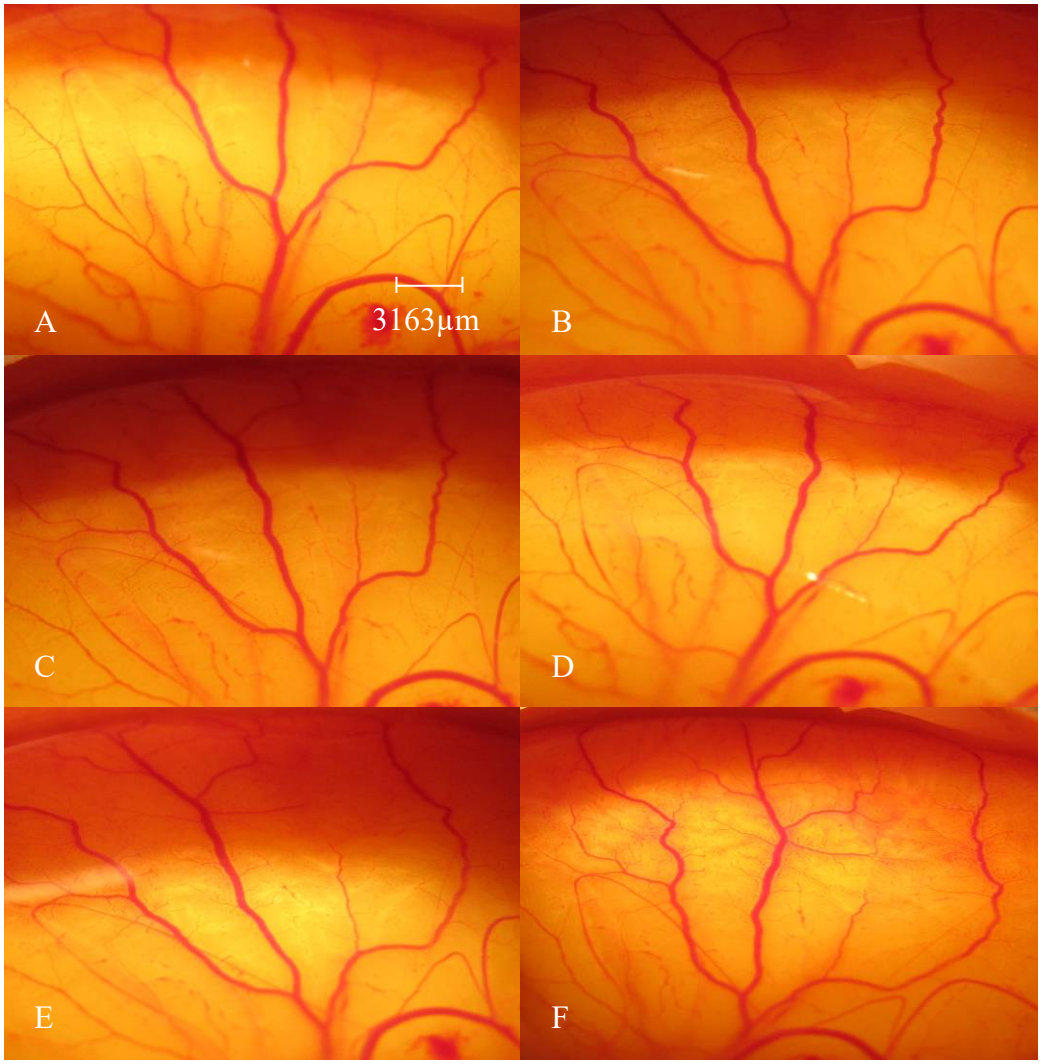

Figure 4. Sequence of photographs illustrating the effect of 2ml of unmedicated gel at pH7.8 on the membrane over a 5-minute period. A. Healthy membrane at T_0, B. Membrane at $T_{0.5minutes}$, C. Membrane at $T_{1minute}$, D. Membrane at $T_{2minutes}$, E. Membrane at $T_{4minutes}$, F. Membrane at $T_{5minutes}$.

The medicated gel at pH7.4 produced no discernable visual changes to the membrane (Figure 5).

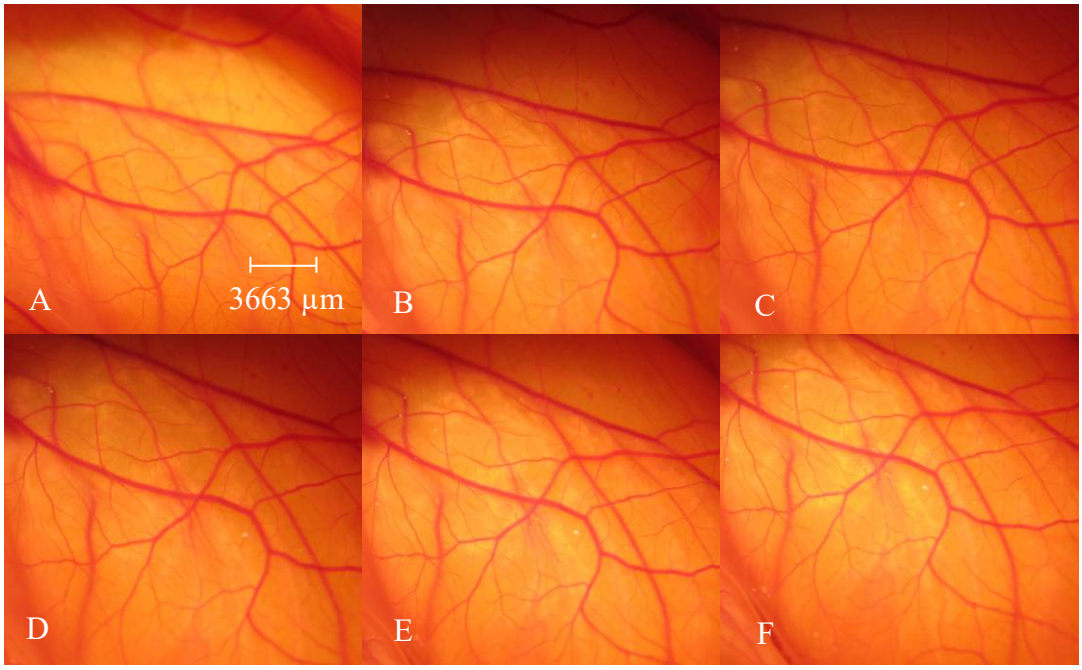

Figure 5. Sequence of photographs illustrating the effect of 2ml of medicated gel at pH7.4 on the membrane over a 5-minute period. A. Healthy membrane at T_0, B. Membrane at $T_{0.5minutes}$, C. Membrane at $T_{1minute}$, D. Membrane at $T_{2minutes}$, E. Membrane at $T_{4minutes}$, F. Membrane at $T_{5minutes}$.

The medicated gel at pH4.5 caused minor haemorrhage (figure 6).

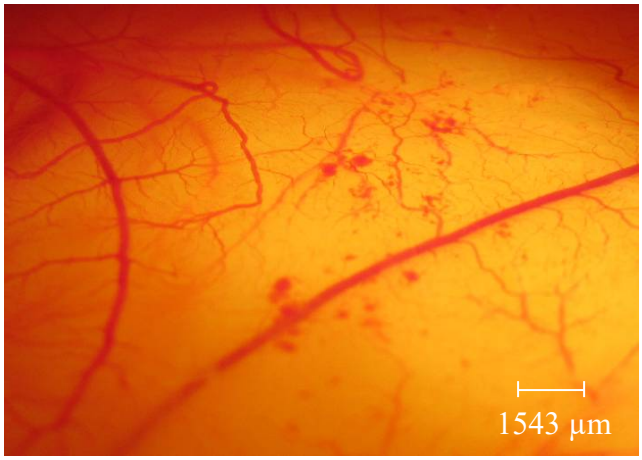

Figure 6. The medicated gel at pH4.5 produced a minor haemorrhage over a small area of the membrane.

The unmedicated gel at pH7.4 produced no visible damage (results not shown). The medicated solution at pH6.45 caused no haemorrhaging, however it is interesting to

note that there was a marked swelling of the yolk and vitelline membrane (results not shown). This 'turgidity' effect was caused by the hypotonic nature of the solution, where the cell contains a higher concentration of solutes than the solution, and water moves into the cell causing swelling and pain in the eye[52]. This solution was made as per the formulation of the current eye drop solution, the pH of which was found to be higher than the published value of 4.5 at 6.45[53]. The irritation experienced by patients could be due more to the hypotonic nature of the solution, and subsequent swelling of the ophthalmic tissues, rather than the low pH of the drops[54]. As a comparison, the same ophthalmic solution was tested with a pH of 4.75.

The medicated solution at pH 4.75 caused minor haemorrhaging and turgidity of the yolk and vitelline membrane, as well as marked hyperaemia (Figure 7). Hyperaemia is an inflammatory response to an irritant, and the process is similar to that of injured rabbit conjunctival tissue[20].

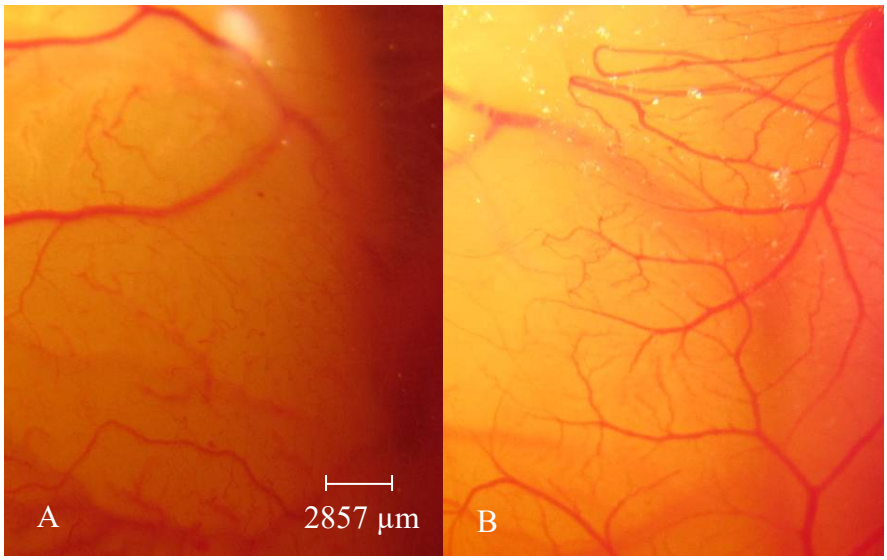

Figure 7. A. Healthy membrane before application of the cysteamine solution, B. The low pH and hypotonic nature of the current eye drops caused swelling and minor haemorrhaging, as well as hyperaemia.

The test solutions can be semi-quantitatively graded using the method developed by Gupta et al[55]. Each test solution was assigned a grade depending on how much haemorrhaging was visible (table 1). A score of zero was obtained for saline, and NaOH recorded a score of 3 (table 2).

Table 1. Scoring scale for the HET-CAM test[55].

Effect	Score	Description
No visible haemorrhage	0	Non irritant
Just visible membrane discolouration	1	Mild irritant
Structures are covered partially by haemorrhage	2	Moderate irritant
Structures are covered completely by haemorrhage	3	Severe irritant

Table 2. Scores obtained from the HET-CAM test.

Test solution	Score
Saline 0.9% w/v (negative control)	0
1M NaOH (positive control) pH14	3
Unmedicated gel pH7.4	0
Unmedicated gel pH7.8	1
Medicated gel pH4.5	1
Medicated gel pH7.4	0
Medicated solution pH6.45	1
Medicated solution pH4.75	2

This semi-quantitative scoring method is still partially subjective, but does allow a number to be assigned to each test solution[16]. The medicated solutions both scored higher values despite a lack of haemorrhage due to the turgidity of the yolk and vitelline membrane.

In order to fully quantify the photographic results, the images were subjected to software analysis using Photoshop® and ImageJ. The data was analysed and processed as a 'grey value' for each pixel along a uniform length (0 = black, 255 = white) i.e. a lower number correlates to more haemorrhaging (Figure 8). All five replicates were analysed, and the averages plotted with standard deviations (Figures 9 and 10).

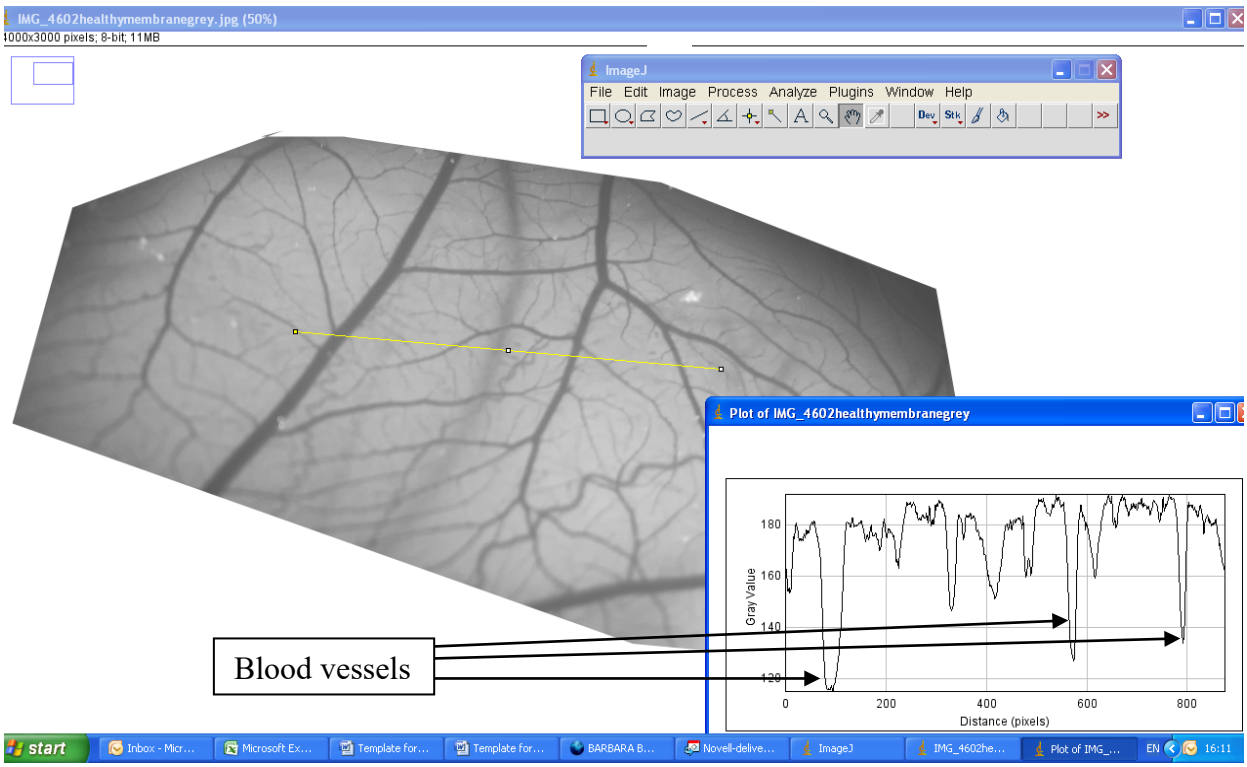

Figure 8. An example profile plot on ImageJ, highlighting the correlation between dark blood vessels and lower greyscale values.

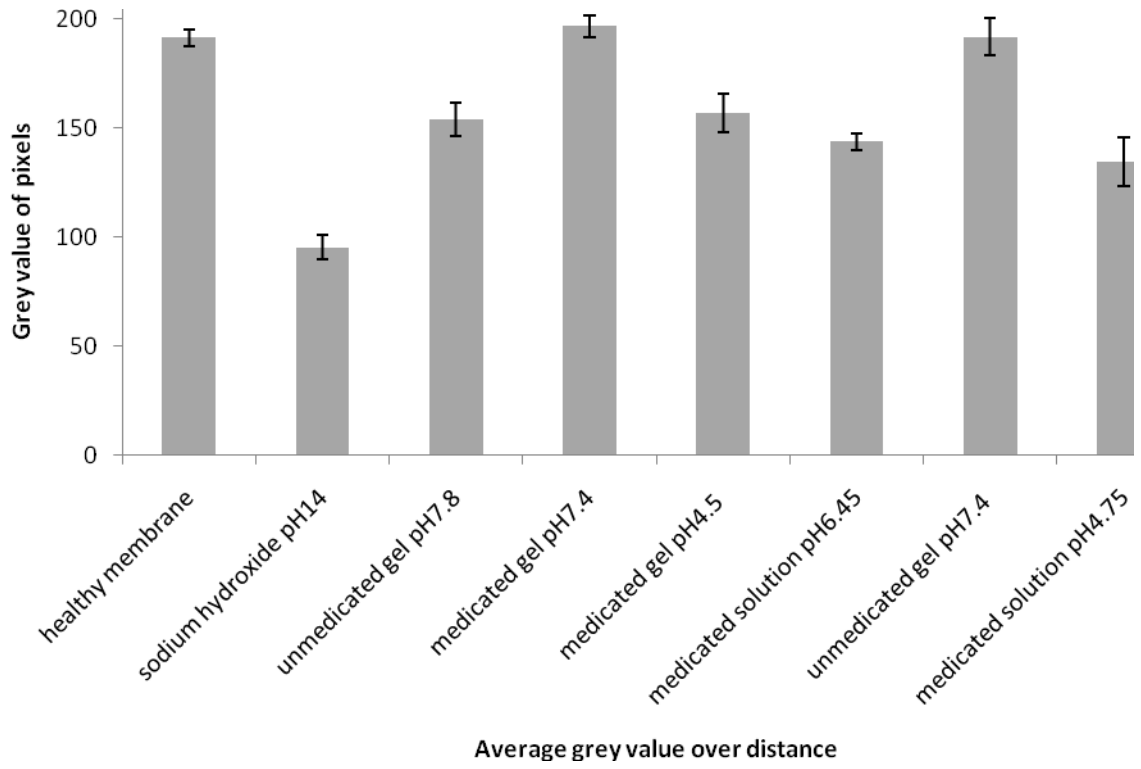

Figure 9. Bar chart of the average grey value over distance for each of the test solutions (n = 5, ±SD).

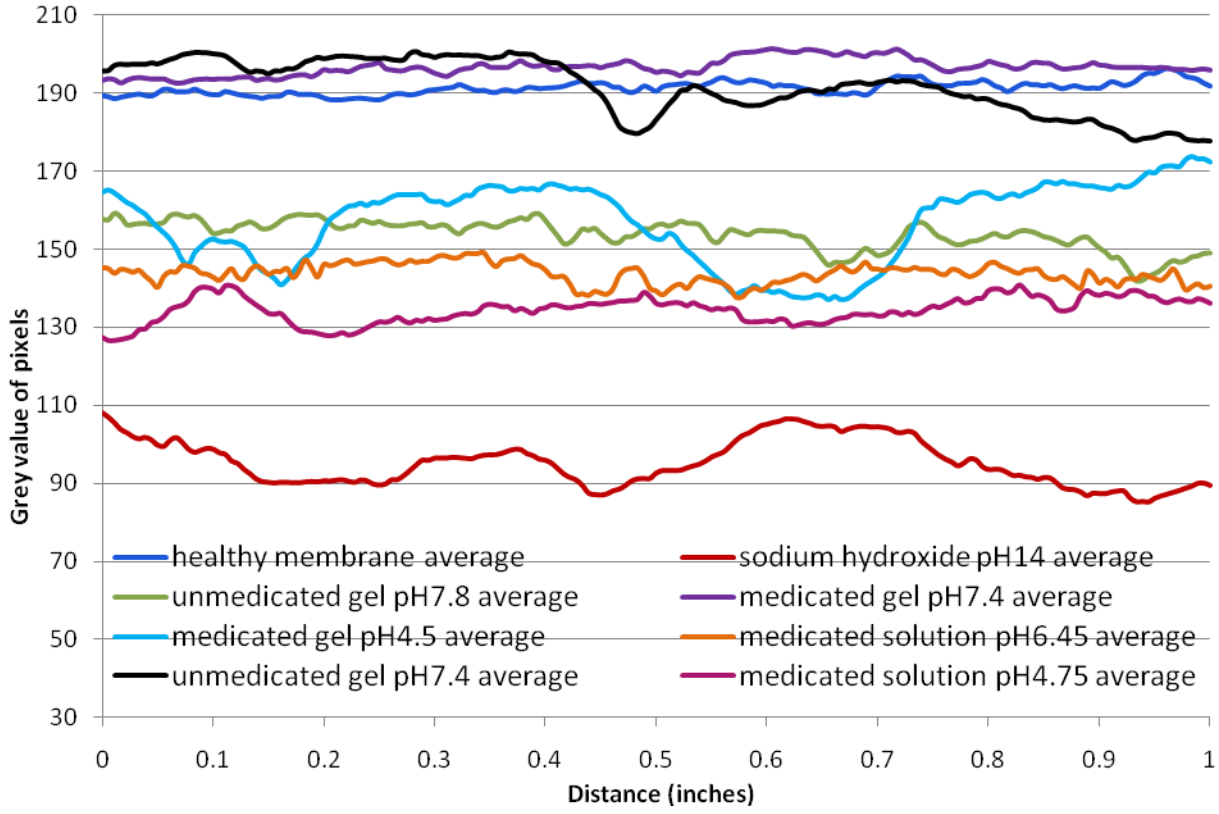

Figure 10. Plot of the grey value over distance for each of the test solutions.

The current ophthalmic solution has a pH beyond the limits of ocular tolerance of 6.6-7.8[48-51] which produces minor eye irritation. The HET-CAM tests demonstrate that the ophthalmic solution is also hypotonic, and causes discomfort from tissue swelling. As a result of these factors, and the chronic and laborious dosage schedule, compliance with the treatment can be poor. The long-term outcomes of this non-adherence to treatment can be serious, with blindness a potential end-point.

The Organisation for Economic Co-operation and Development (OECD) Guideline 405, entitled 'Guidelines for the testing of chemicals; Acute eye irritation/corrosion' states that, "Substances that have demonstrated corrosive or severe irritant properties in an *in vitro* or *ex vivo* test that has been validated and internationally accepted for the assessment specifically of eye corrosivity/irritation, need not be tested in animals. It can be presumed that such substances will produce similar severe effects *in vivo*". The test has been validated by an EU Directive on dangerous substances.

The HET-CAM test is useful as a model for ophthalmic tissue (such as the conjunctiva), since it is a functional membrane complete with vasculature and inflammatory responses, and can be evaluated for endpoints that are associated with ocular injuries. The HET-CAM test evaluates the ability of a test substance to damage blood vessels, and cause haemorrhage, coagulation, hyperaemia or lysis. The endpoints evaluated in the HET-CAM test method are different to those evaluated in the *in vivo* test method (discharge from the conjunctiva, redness and chemosis), but are thought to correlate well to the mechanisms of toxicity that could cause these *in vivo* endpoints.

Of the three analysis methods used in this paper, qualitative, semi-quantitative and quantitative, none fully describes the effects of all the test solutions. It is useful to include all three methods to allow a full and accurate description of the damage caused to the CAM. The study demonstrates that the new gel formulation does not cause damage to the membranes of the CAM.

4.0 Conclusions

A new ophthalmic gel formulation has been developed to treat the corneal crystals associated with cystinosis. As part of characterisation tests within our laboratories, the ocular irritation potential of the gel was determined using the HET-CAM test. The HET-CAM test uses the functional vasculature of the chicken's placenta to represent the ophthalmic situation *in vivo*. The new formulation outlined in this paper offers the possibility of a reduced daily dosage schedule (once or twice a day) and reduced ocular toxicity (zero score, non-irritant), which may improve patient compliance. This in turn may improve long-term morbidity. The HET-CAM tests demonstrate that the new formulation is safe for *in vivo* use.

Acknowledgements

The authors gratefully acknowledge support from Sparks, The Rooney Foundation and Robert Gordon University, Institute of Health and Welfare Research.

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