Summary of Research completed for Waikato Medical Research Fund Grant Application

Our preliminary work with the retinal ischaemia reperfusion model in the rabbit eye encouraged us that we had a reliable model, albeit based on a very small number of animals (for cost reasons).

We also wished to investigate the potential to improve the study by implanting NTCELL prior to the ischaemia reperfusion injury. This was hypothesized to allow the NTCELL to 'acclimatize' and be fully functional prior to the retinal injury.

Unfortunately, after a considerable amount of work, we have come to the conclusion that the retinal ischaemia reperfusion model in the rabbit is unreliable. Our initial results suggesting it was very reliable were not confirmed when we used more animals. With more animals we found that the rabbit eye to have a propensity for recovery from an ischaemia reperfusion injury equal to or in excess of those published. We looked at controlling other potential factors such as the age of the animal, without success. Furthermore, it would appear that the publication we used for our methods likely contains false data. In retrospect this publication is contrary not only our findings but to comparable data in humans. From a statistical perspective the data in the reference paper are very unlikely to be correct - the data are too good to be true.

We did also complete limited work on the concept of pre-implantation. This work suggested that NTCELL are adversely affected by the period of high intraocular pressure required for inducing a retinal ischaemia reperfusion injury.

Due to the problems experience with the model we did not proceed with all tissue analysis. We did limited vitality testing of retrieved NTCELL with useful information gained. We did undertake some preliminary histology. We had some work done by the histology laboratory at Auckland Medical School. This involved gaining experience with normal rabbit eyes and then moving on to some of the study eyes. However we discontinued histology analysis once was obviously model would not work. For the same reasons no vitreous analysis was performed. No work analysis of the lenses was performed (although these had been collected and stored).

In contrast to the rabbit it is very well established that the rat retina ischaemia reperfusion model is reliable. I have previously done some work with rat but as the lens fills all but 0.2 ml of the rat eye this is technically very difficult and impractical for implantation of NTCELL. That was why we moved to the rabbit eye. I am currently revisiting the possibility of using the rat and have commenced preliminary work on developing an aphakic (lens removed) rat. This will create sufficient space for implantation of NTCELL. However, there are some difficulties with this. Removing 95% of the eye volume is a major insult and I am currently looking at ways to improve recovery. Should we obtain a successful aphakic rat eye then the retinal ischaemia reperfusion model has been used successfully by Prof Colin Green in Auckland. This would allow me to tap in to local experience for a reliable aphakic rat retinal ischaemia reperfusion model that can be used to assess NTCELL intraocular implantation. We are also commencing some work assessing the effects of the neonatal Auckland Island pig choroid plexus secretome (as opposed to NTCELL implantation per se) on a model of retinal degeneration.

There is still very good reason to believe that choroid plexus secretome and/or intraocular NTCELL will protect from retinal degenerative disease. This is because of the work done using NTCELL on models of three CNS degenerative diseases and clinically on Parkinson's disease. There has been further progress using NTCELL brain

implantation for human CNS degenerative disease - late stage Parkinson's disease. Two successful human implants have been performed in Auckland and another two patients will be implanted shortly to complete the phase 1 trial. As the retina is CNS tissue the potential for translation of CNS preclinical and clinical work to retinal disease is conceptually appealing.

I know that there has been concern expressed by the medical committee regarding the possibility of transmitting disease from pig to other species via xeno-transplantation of pig choroid plexus. Following your request for clarification we provided supplied further information regarding the measures that have been taken to address this issue. I would like to reiterate that there has been both extensive work done to confirm that the Auckland Island pig is free of disease and extensive review of this work by a number of regulatory and ethical bodies prior to the commencement of clinical programs for both the islet cell implant (DIABECELL) for diabetes which is now in phase 3 trial and the choroid plexus cell implant (NTCELL) program which is now in phase 1 trial for Parkinson's disease.

Specific Aims of the Project

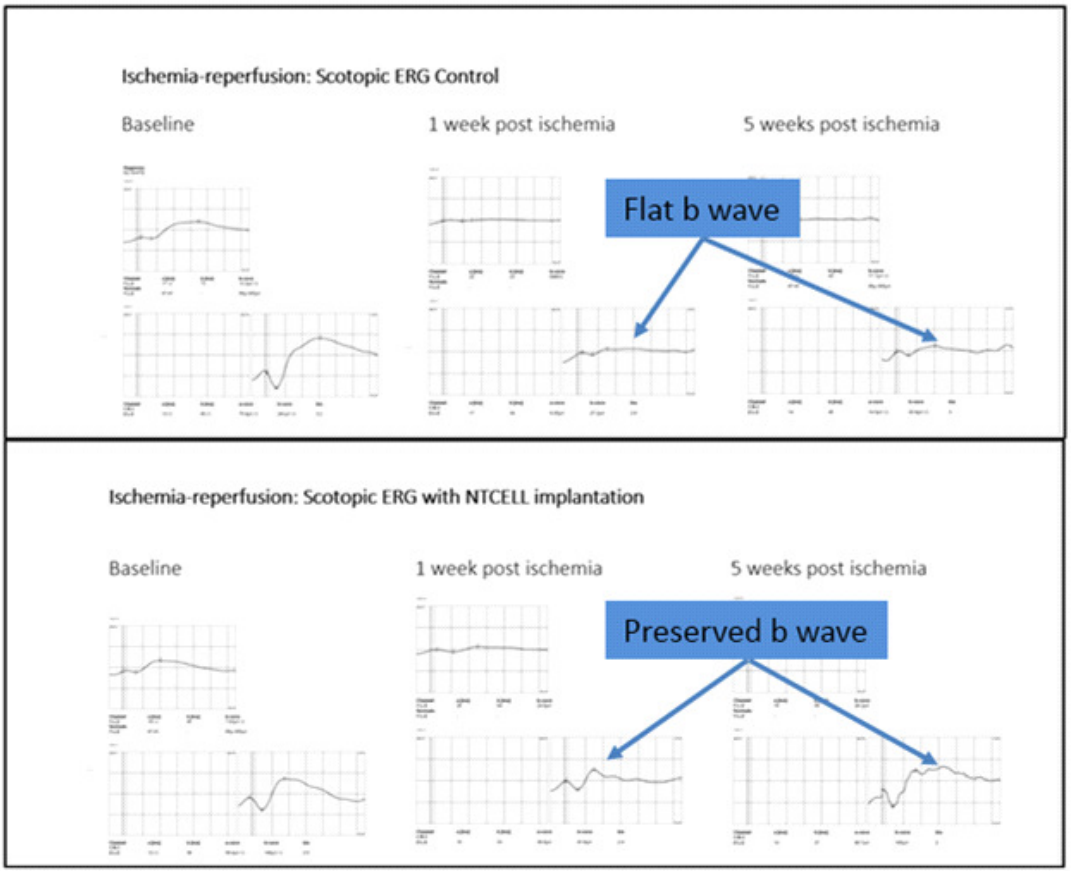
Primary aim

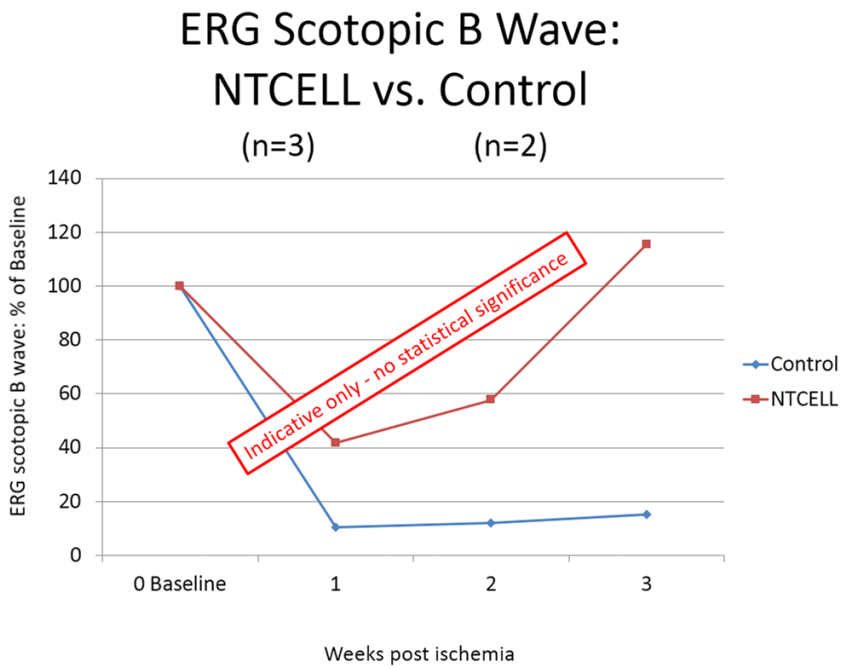
To test the effect of intravitreal implantation of encapsulated neonatal choroid plexus on the rabbit model of in the retina neurodegeneration retinal ischaemia reperfusion model

Secondary aims

- Assess the effect of NTCELL on cataractogenesis
- Provide longer-term data on the rabbit retina ischaemia-reperfusion model

Preliminary work with Ischemia-reperfusion model and NTCELL implantation





Research design

Thirty adult (3 month old) New Zealand white rabbits randomly assigned to five groups of six animals. The left eye is the study eye, the right eye is untouched:

1. Ischaemia-reperfusion only	(Positive control)

Implantation immediately post ischaemia-reperfusion

2. Ischaemia-reperfusion plus empty capsules	(Vehicle control)
3. Ischaemia-reperfusion plus NTCELL capsules	(Treatment)

Implantation prior to ischaemia reperfusion (preconditioning)

4. Empty capsule preconditioning then ischemia-reperfusion	(vehicle control-preconditioning)
5. NTCELL preconditioning then ischaemia-reperfusion	(treatment-preconditioning)

Analysis Plan

Electroretinography (ERG)

A baseline ERG prior to ischemia-reperfusion and at week 1, 2, 3, 5, 8, 11 and 14 after ischemia-reperfusion +/- surgery.

Tissue analysis

Lens

HPLC analysis of lens samples

NTCELL and empty capsules

NTCELL and empty capsules retrieved from implantation study eyes in groups 3 and 4 for microscopic examination, and NTCELL-viability and Enzyme-linked immunosorbent assay (ELISA) or immunoperoxidase testing.

Vitreous

ELISA or immunoperoxidase testing for proteins of particular interest.

Retina histology

Retinal and choroidal thickness and layer thickness –especially inner retinal layers- is made using either H&E and Nissl or toluidine blue

Immunocytochemistry will be done for Glial fibrillary acidic protein (GFAP).

Statistics

To compare study groups and control eyes. The effect of the implants will be determined by comparison of the ERG, retinal and vitreous testing of the study groups and control eyes.

PART 1 DP1321

EXPERIMENTAL DESIGN:

Further establishment of consistent Retinal Ischaemic Reperfusion injury in New Zealand White Rabbits as a model of Retinal Degeneration

Retinal Ischaemic Reperfusion Injury Model

1. Rabbits anaesthetized using ketamine/xylazine (intramuscular)

2. Left eye sterilized with ½ strength povidine-iodine

3. Human speculum used to expose the eye by holding the eye lids open

4. Small 1mm conjunctival incision made to gain access to the vitreous cavity and a 25Ga infusion cannula attached to a saline bag inserted

5. Saline bag attached at a height of 2.04m above the height of the rabbit eye to generate an intraocular pressure of 150mmHg

6. Cessation of retinal perfusion is confirmed by opaque appearance of the eye

7. Increase intraocular pressure is released after 60mins and confirmed by the return of normal eye colouration when at normal pressure (20mmHg)

Electroretinography (ERG)

A light stimulus based electrical output used to assess retina activity

Animals are dark adapted overnight

Ketamine/Xylazine is delivered intramuscular to place the animal under general anaesthesia

Tetracaine eyedrops are delivered to the eye immediately prior to conducting the ERG to provide a local anaesthetic to the animal

Tropicamide 1% and phenylephrine 10% eyedrops to dilate the pupils

Animals then placed into the ERG machine

Short flashes of light then stimulate the photoreceptors and electrical output detected by the electrodes

ERG schedule: Baseline prior to retinal ischaemia and 1, 3, 5 and 10 weeks post initial injury

EXPERIMENTAL ANIMALS:

24 week male New Zealand White Rabbits weighing 3.69 and 3.65kg at the commencement of the study. Initially "young" rabbits of approximately 14-16 weeks approximately 3kg were sourced from an external experimental rabbit breeder. However, due to time constraints and availability, it was not possible to commence until a couple of months following their arrival.

RESULTS:

Retinal ischaemia reperfusion injury was induced in two New Zealand White Rabbits and monitored for 3 weeks using ERG. These initial results demonstrated a significant decrease in the b-wave response typically observed in animals which have been subjected to significant durations of retinal ischemia. The percentage change of the b-wave from baseline over 10 weeks for the first two rabbits following the ischaemia induced injury is shown in Figure 1.

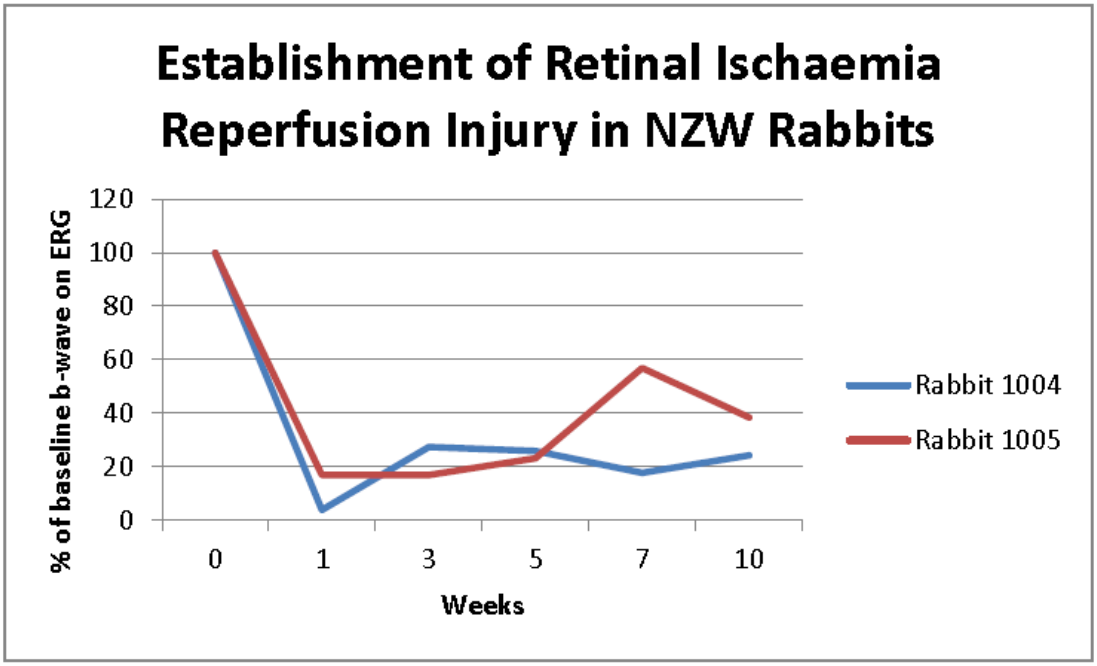


Figure 1. Percentage change of b-wave from baseline over 10 weeks, Ischaemia Only Group

In the week immediately following ischaemia reperfusion injury, there was an approximate drop in b-wave response of approximately 80%. This decrease was consistent over the 10 weeks following with no significant recovery of the b-wave. Histology of the retinal of the eye is required to confirm loss of retinal ganglion cells.

CONCLUSION:

Based upon ERG findings, we are able to generate a reliable model of retinal ischaemia reperfusion injury in NZ white rabbits. We therefore believed we could reproducibly generate this model for the evaluation of NTCELL and its ability to rescue intraocular pressure induced retinal ischaemia reperfusion injury in NZ white rabbits.

PART 2 DP1321

NTCELL/empty capsule implantation/sham surgery

1. Vitrectomy of the vitreous cavity is performed using a vitrectomy machine. Two 25Ga cannulas are inserted 1mm posterior to the limbus. A vitreous cutter is inserted into one cannula and used to remove vitreous. A light pipe is inserted into the second cannula and used to guide the removal of the vitreous

2. NTCELL/empty capsules are prepared for implantation into the space created by the vitrectomy (step omitted for sham surgery)

3. Infusion cannula opening is extended to allow capsules to be implanted using a 20Ga catheter (step omitted for sham surgery)

Capsule Implantations:

Approximately 1500 capsules in total 1mL saline in a 1mL syringe

Capsules pre-washed in 3xHBSS in a 6 well plate, followed by 1xsaline

Capsules drawn up in fresh 1mL saline for implantation

4. Closure of sclerotomies with 8.0 vicryl.

EXPERIMENTAL ANIMALS:

The first two control animals were included as part of the control group of animals for continuing the study. NTCELL and Empty capsule implanted groups of animals' commenced immediately following confidence that a model had been established. Animals purchased for the component of the study were of approximately 14-16 weeks of age by the time they were used for experiments. Groups of animals are summarized below.

Table 1.

CONDITIONS	NUMBER OF RABBITS
Ischaemia Only	6
Ischaemia + Vitrectomy	6
Ischaemia + Vitrectomy + Empty Capsules	6
Ischaemia + Vitrectomy + NTCELL Capsules	6
Total Number of Rabbits	**24**

RESULTS:

ERGs:

The first three NTCELL implanted animals showed a remarkable recovery of ERG. These results and for the initial two control animals were included in the grant submission to WMRF.

More animals were entered into the pre-grant study. However, the results of completing more control and treatment animals did not show a similar trend. Inclusion of all animals implanted with NTCELL or receiving the ischaemia only injury resulted in little difference between the two groups (Figure 2). An initial suppression of the b-wave was observed in both groups after one

week. Interestingly, when including younger and older rabbits, the average b-wave was only 50% of that observed at baseline.

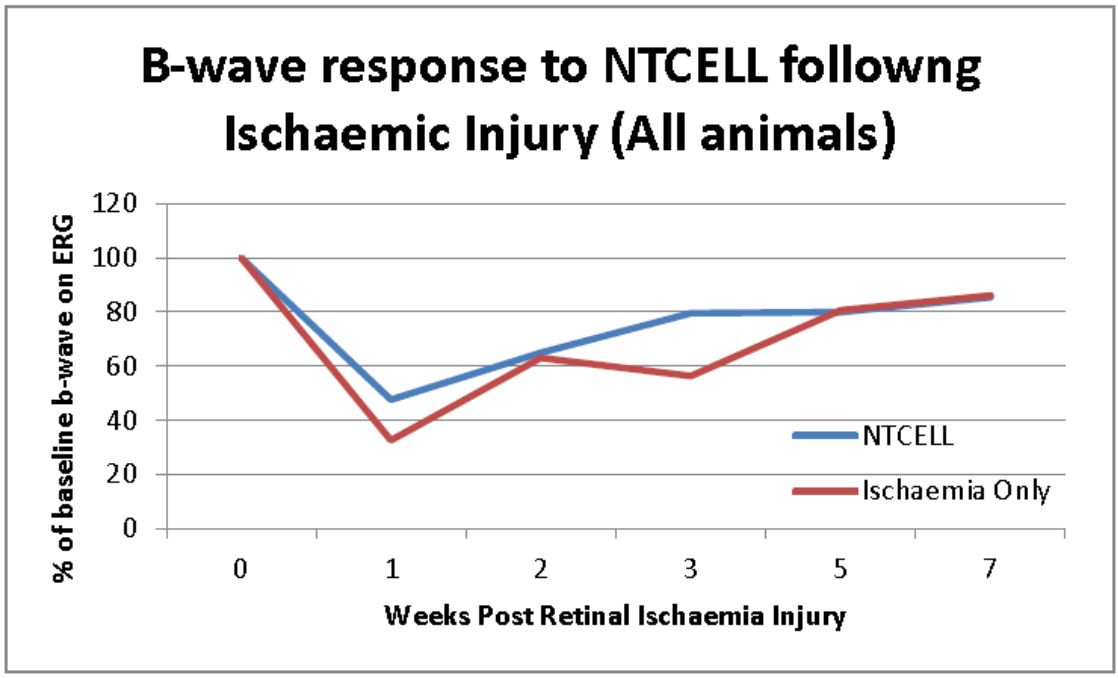


Figure 2. ERG b-wave percentage from baseline following ischaemia reperfusion injury, includes all animals

Analysis of only the 14-16 week rabbits demonstrated that these animals were less susceptible to retinal ischaemia reperfusion injury compared to the two older rabbits 1004 and 1005 (Figure 3). This is evident by a suppression of the b-wave from baseline of only approximately 50%, not 90% which was observed with the two 23 week old rabbits used for the first two controls.

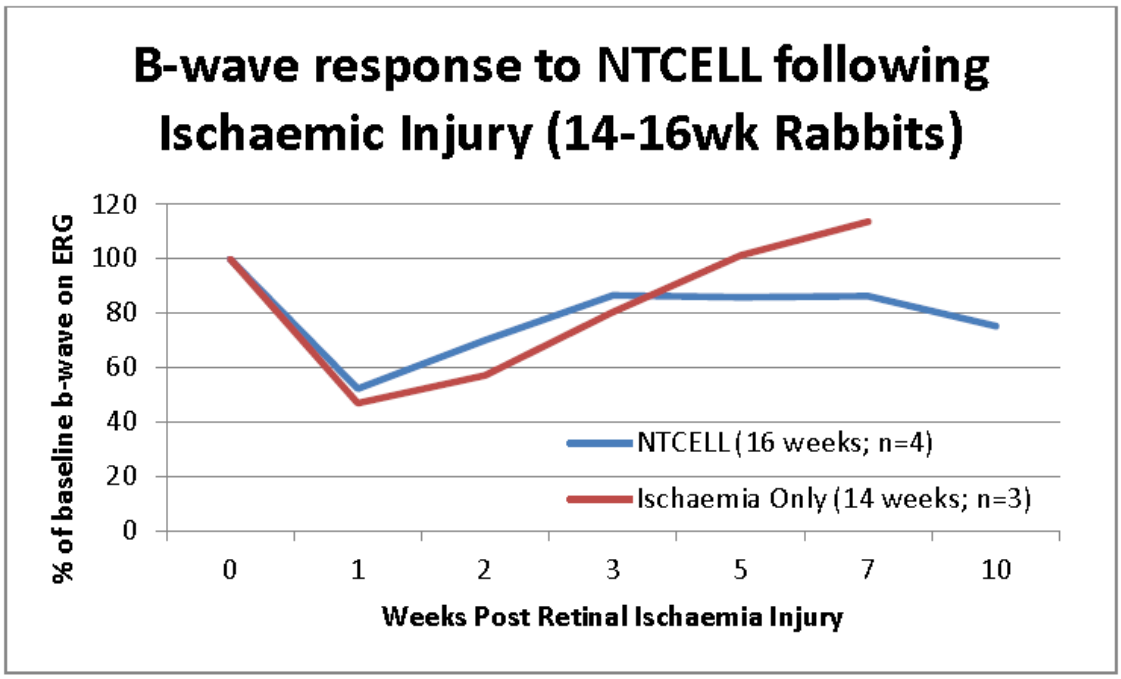


Figure 3. ERG b-wave percentage from baseline following ischaemia reperfusion injury of only rabbits aged 14-16 weeks at time of ischaemia reperfusion injury

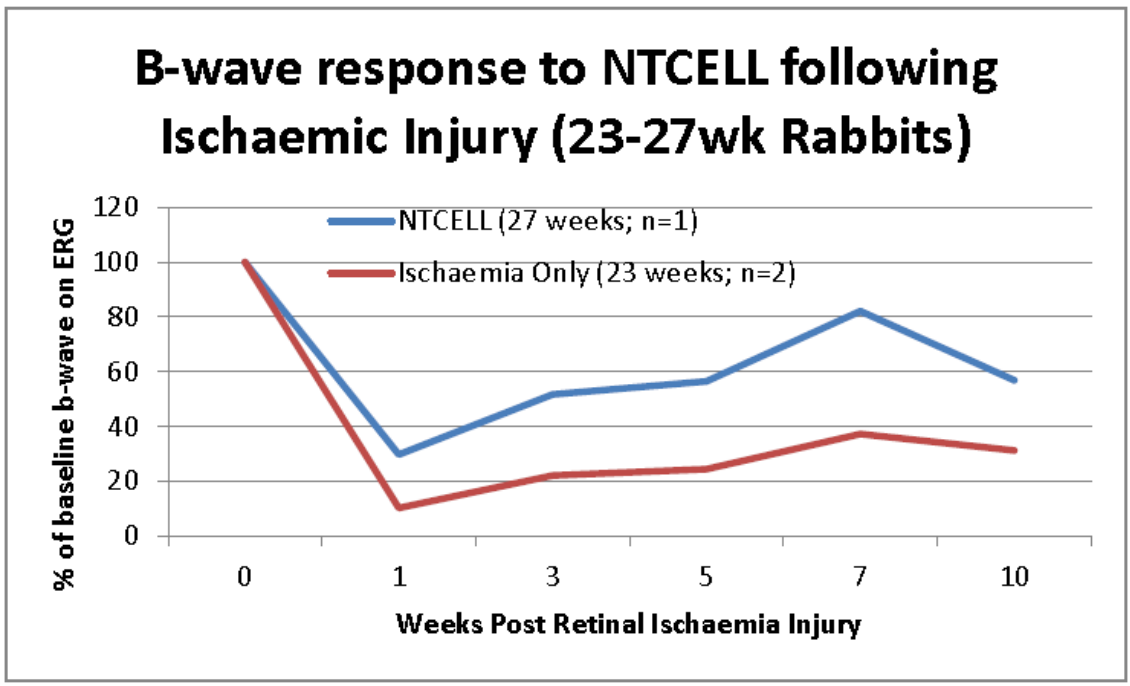


Figure 4. ERG b-wave percentage from baseline following ischaemia reperfusion injury of only rabbits aged 32-27 weeks at time of ischaemia reperfusion injury

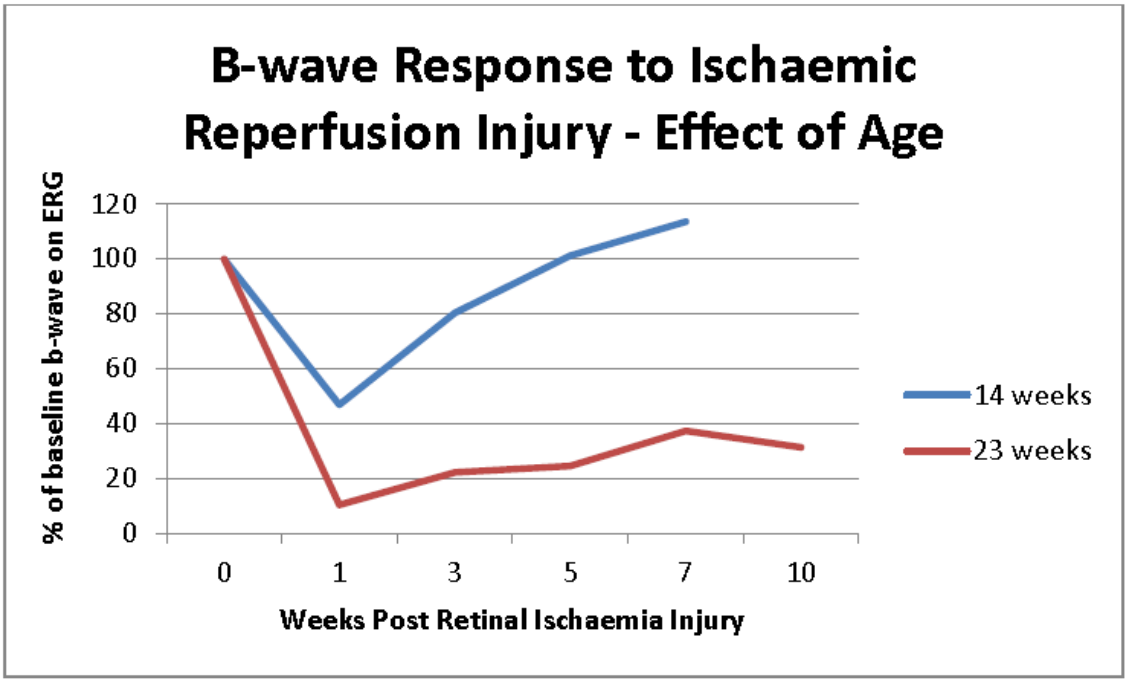
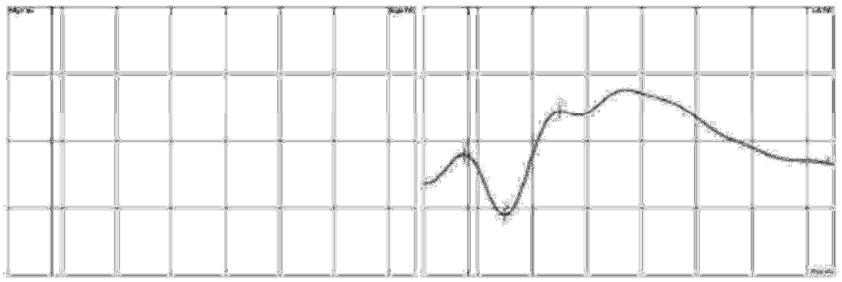


Figure 4. ERG b-wave percentage from baseline following ischaemia reperfusion injury of rabbits by week's age at time of ischaemia reperfusion injury

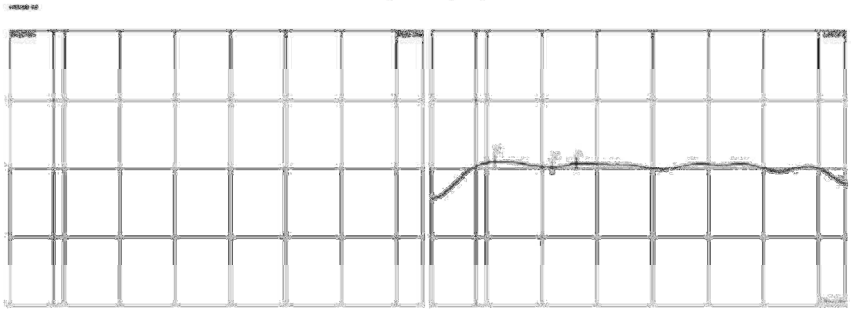
Rabbit 1004 – Ischaemic Injury Only

Baseline



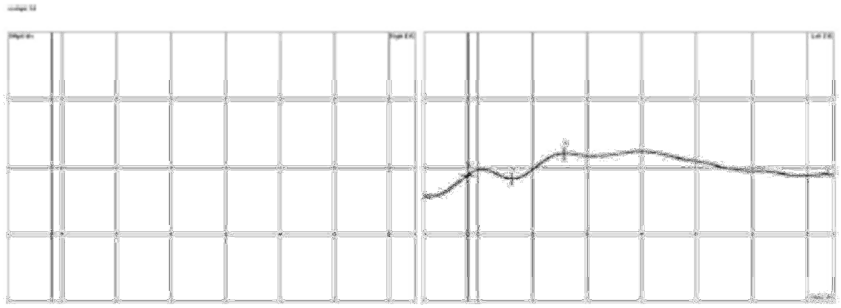
Channel	a [ms]	b [ms]	a-wave	b-wave	b/a
1 R-1					
2 L-2	14 (!)	34	89.7µV (!)	154µV (!)	1.7
Normals	14-22	33-46	155µ-356µV	290µ-654µV	

1 week post injury



Channel	a [ms]	b [ms]	a-wave	b-wave	b/a
1 R-1					
2 L-2	28 (!)	56	6.61µV (!)	6.74µV (!)	1.02
Normals	14-22	33-46	155µ-356µV	290µ-654µV	

10 weeks post injury



Channel	a [ms]	b [ms]	a-wave	b-wave	b/a
1 R-1					
2 L-2	16	36	5.46µV (!)	37.2µV (!)	6.8
Normals	14-22	33-46	155µ-356µV	290µ-654µV	

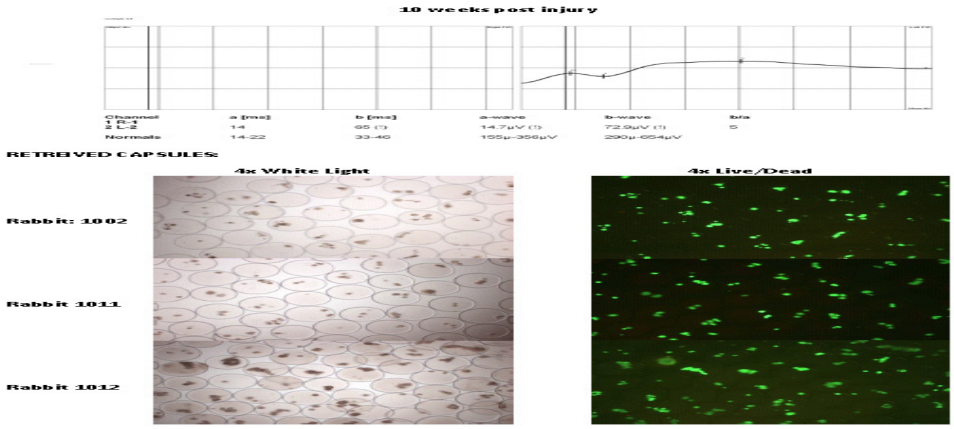


Figure 5. ERG scotopic b wave

Approximately 50% of the capsule implanted into the vitreous cavity was retrieved. This is involved careful dissection of the eye globe followed by removal of the cornea, iris and lens tissues. Remaining vitreous gel was removed using a 3mL transfer pipette. Any capsules in the vitreous gel was gently removed and free floating capsules were gentle washed from the cup of tissue containing the retina, retinal pigmented epithelium and associated tissues. Capsules were washed with HBSS and collected into a 50mL falcon tube and transported from Ruakura Research Centre back to Living Cell Technologies for further analysis. Capsules were stained with LIVE/DEAD® Cell Viability Assays (Life Technologies - Invitrogen) and imaged using a fluorescent microscope.

Retrieved capsules were all intact, remained structurally similar to the capsules that were initially implanted. Encapsulated choroid plexus cellular materials were observed in the majority of capsules. Live/dead staining revealed that the cellular material contained in these capsules were alive, as evident by the green staining, in contrast the amount of dead/dying material in the capsules was very low as seen by the absence of red staining material.

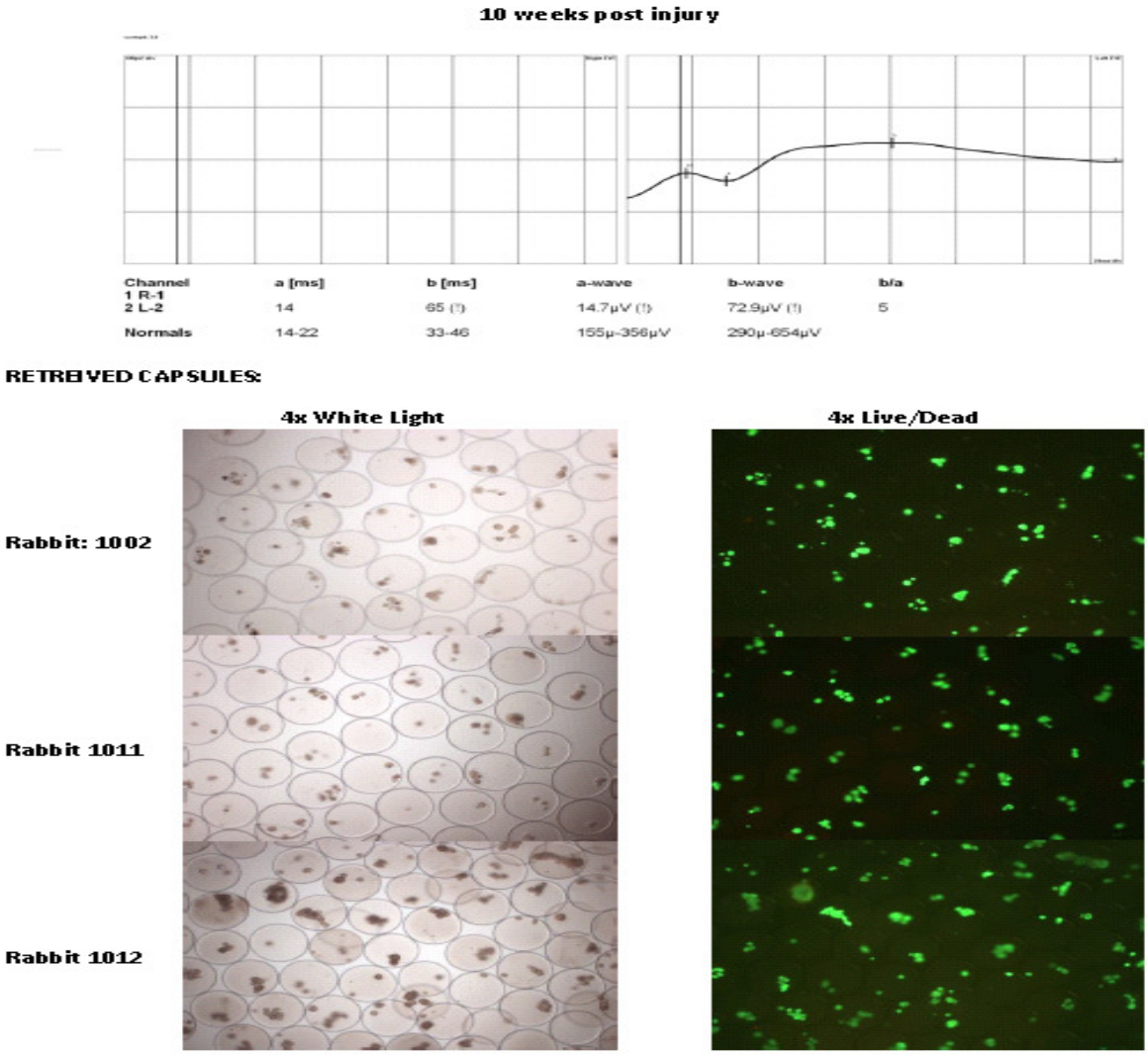


Figure 6. NTCELL viability test results

DISCUSSION:

Although a well described model in rats, this model in rabbits is not as widely used. For our purposes, where encapsulated choroid plexus clusters, NTCELL, are required to be implanted, we require a relatively large animal to perform this analysis. As a consequence it was considered important to determine if we can reproducibly generate this injury model in rabbits. Two rabbits were used for this purpose and resulted in suppression of the b-wave from baseline of approximately 80-90% 1 week following injury and was maintained over the following 8-10 weeks. Due to factors beyond our control, rabbits that were 14-16 weeks of age at the time of purchase but were 24-26 weeks of age by the time the injury was induced.

Subsequent experimental procedures were performed with 14-16 week old rabbits. From the results obtained it showed that age to be a significant factor in successfully inducing a reproducible model which did not recover over time. 1 week after injury, younger rabbits only have a reduction in b-wave amplitude from baseline of approximately 40-50%. This suggests that these younger rabbits are less susceptible to injury from this type of insult.

As a consequence of the response of the younger rabbit control animals, interpretation of the results obtained from the animals which received NTCELL implantations into the vitreous cavity following vitrectomy is difficult. Firstly, a direct control was not completed due to availability of rabbits, this was the ischaemia injury + vitrectomy groups of animals. Although we observed a similar improvement in the b-wave response in NTCELL treated animals and ischaemia only groups. NTCELL treated animals were subjected to additional vitrectomy procedure which ischaemia only animals did not. What contribution (if any) of the vitrectomy is difficult to determine without having this additional groups. The vitrectomy in addition to the ischaemia injury may have induced a greater insult than that observed with ischaemia alone. Of interest, Kawai described a number of significant factor s which influenced the success of the ischaemia reperfusion injury in rat models [2]. Importantly, it was the effect of age which was the single greatest contributor to a rat's susceptibility to retinal ischaemia reperfusion mediated injury. This is summarized in the table 2 below.

	2 year rats	2 months rats
Central Retina	25% fewer RGC in 2 year old rats	
Peripheral Retina	40% fewer RGC in 2 year old rats	
+ Ischaemia in Central Retina	28% loss in 1wk	15% loss in 1wk
+ Ischaemia in Peripheral Retina	38% loss in 1wk	21% loss in 1wk

Table 2.

Older rats of approximately 2 years inherently have fewer retinal ganglion cells in both the central and peripheral regions of the retina compared to 2 month old rats. Furthermore, when subjected to ischaemia injury, there is a greater rate of RGC loss in 2 year old rats compared to 2 month old rats, approximately twice as many RGC are lost in the older rats [2].

Importantly, 16-30 week old rabbits are considered "young adult" and reaches sexual maturity at 4-6 months of age. Furthermore, retinal degeneration and related conditions are considered conditions of the "elderly". Therefore, the use of younger rabbits in the age range of 14-16 weeks should not be used for our experiments. We should consider using rabbits which are at least 24-30 week for induction of ischaemia as a more reliable and reproducible model.

Capsules retrieved at post mortem appeared in excellent condition and choroid plexus clusters were viable. However, this does not provide any clues as to functional capacity of these capsules in vivo. In future studies, retrieved capsules could be subjected to the VEGF assay to determine if these encapsulated CP clusters are capable of secreted VEGF. This could be normalized against measured DNA in these capsules. However this procedure will destroy cellular integrity resulting in an inability to perform histological analysis. It might be more useful if VEGF secretion was measured to determine if the CP clusters are capable of secreting VEGF and then the capsules are prepared for histology without performing any measurements of DNA.

In addition to the complicating factors of age and the effects of vitrectomy, a number of technical difficulties were required to be overcome for the procedure to be considered a success. These include:

1) Avoiding contact with the very large lens in the rabbit which can lead to the development of cataract.

2) Delivering a known number of capsules into the vitreous cavity

CONCLUSION:

Rabbits did not recover from ischaemia reperfusion injury after 3 weeks and therefore it was considered a reliable model to continue with additional treatment groups including NTCELL implantations. However, the age of these rabbits was approximately 24 weeks of age due to delays in the experimental protocol resulting in stock animals remaining unused to significant length of time.

Further work:

· Small experiment to determine if increasing the age of the rabbits provides a reliable ischemia reperfusion model

· Complete the ischaemia + vitrectomy group to compare the effect of NTCELL implantation with the direct control group

· Sodium Iodate intravenous delivery to induce an alternative model has been considered but not given top priority for this initial body of work

REFERENCES

1. Abdallah W, et al., *Vitreal oxygenation in retinal ischemia reperfusion.* Investigative Ophthalmology & Visual Science, 2011 **52**(2): p. 1035-42.
2. Kawai, S., et al., *Modeling of risk factors for the degeneration of retinal ganglion cells after ischemia/reperfusion in rats: effects of age, caloric restriction, diabetes, pigmentation, and glaucoma.* The FASEB Journal, 2001 **15**(7): p. 1285-7.

PART 3 DP1321

TITLE: Pilot study to determine the potential protective properties of NTCELL pre-implanted into a model of retinal degeneration in rabbits

OBJECTIVE: To conduct a small pilot study consisting of 3 female rabbits approximately 6 months of age to provide preliminary data to determine if pre-implanted NTCELL capsules can survive the ischemic insult and potentially protect against ischemia reperfusion mediated retinal damage.

AIM(s):

- Can pre-implantation of NTCELL can attenuate the magnitude of ischemia reperfusion induced injury in the rabbit?

BACKGROUND/RATIONALE: (see Parts 1 & 2)

Parts 1 and 2 provided initial data which suggested that the parameters used to induce ischemia reperfusion mediated retinal injury was only successful in older rabbits of >22 weeks of age. In younger 14-16 week rabbits, this model of injury was not as reproducible. Furthermore, in the literature it suggests that retinal cells in the eyes of younger rabbits are less susceptible to ischemia reperfusion mediated damage. In this pilot study we will use rabbits of approximately 6 months of age at the time of retinal ischemia reperfusion injury. Furthermore, as this is only a small pilot study we wish to explore the potential protective effect of NTCELL by pre-implanting these capsules into the vitreous cavity 2 weeks prior to the injury. This will allow NTCELL to get acclimatized to the local environment and begin secreting basal levels of neurotrophic factors if the environment permits. Furthermore, the induction of the injury occurs rapidly in the 1hr of high pressure induced ischemia on the eye and the subsequent reperfusion injury. This traumatic injury followed by capsule implantation could be possibly too great to allow for rescue by NTCELL.

EXPERIMENTAL DESIGN:

	d0			d7	d14	D21	
Rabbit 1	Baseline ERG	Vitrectomy	NTCELL	ERG	ERG	ERG	Ischemia
Rabbit 2	Baseline ERG at d21	No Vitrectomy	No implantations	ERG	ERG	Baseline ERG	Ischemia
Rabbit 3	Baseline ERG at d21	No Vitrectomy	No implantations	No ERG	No ERG	Baseline ERG	Ischemia

Table 4.

Capsule Implantations (Left Eye):

· Vitrectomy of the posterior chamber (vitreous cavity)

· 100 capsules in up to 500µL saline

· 20Ga blunt vitrectomy catheter

Ischemia Reperfusion Injury (Left Eye):

· To commence approximately 3 weeks after capsule implantation

· *Cannulate anterior chamber* of the rabbit eye

· *150mmHg pressure for 60mins using saline*

Number of Capsules for Implantation:

For Part 2, we increased the maximum number of capsules into the vitreous cavity following a vitrectomy. Forty, 20 and 10 capsules were implanted for human, non-human primate and rat models respectively of Parkinson's disease. Therefore it was proposed to implant a larger number of capsules as this would maximize the likelihood of observing a positive outcome. We decided to implant 100 capsules.

CAPSULES:

NT012 CPe1

Isolation Date: 24 May 2013

Encapsulation Date: 30 May 2013 (d7)

Received off Production Team: 21 June 2013

Implantation Date: 28 June 2013

RESULTS:

Time Point (Weeks)	b wave Amplitude (µV)		
	A01 NTCELL	A02 Ischemia Only	A03 Ischemia Only
	60 minutes	50 minutes	60 minutes
-3	206	N/A	N/A
0	247 (120%)	193	315
1	264 (128%)	N/A	244 (77%)
2	175 (85%)	NA	249 (79%)

Table 5. ERG readings from 3 female NZ white rabbits which received 60minutes of ischemia reperfusion injury at 150mmgHg into the anterior chamber of the left eye. A01 received NTCELL capsules into the posterior chamber 3 weeks prior to ischemic injury.

A01 – Successful implantation of 100 NTCELL capsules into the vitreous cavity of a female rabbit of approximately 6 months of age following a vitrectomy. This was followed by induction of ischemia reperfusion injury 3 weeks later. It was unusual to observe an increase of 20% in ERG measurements 3 weeks after the initial implantation of NTCELL (Figure 6).

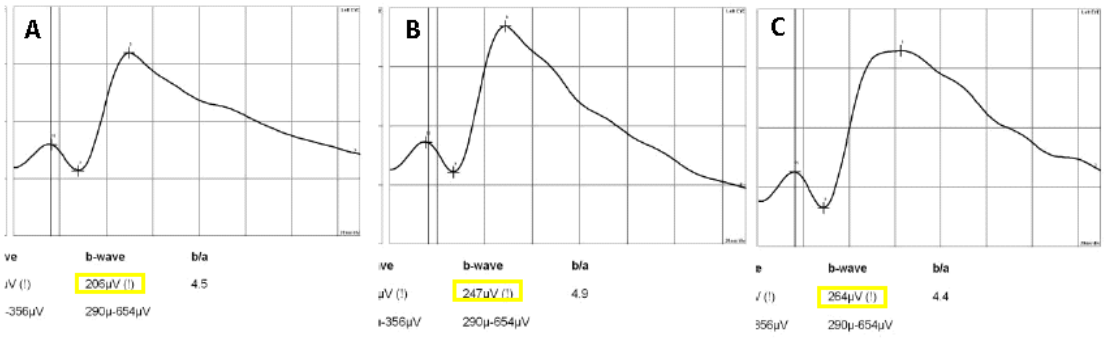


Figure 6. Rabbit A01 ERG profile of the Left Eye from A) baseline prior to vitrectomy & NTCELL implantation B) 3 weeks following vitrectomy & NTCELL implantations prior to ischemia reperfusion injury and C) 1 week following ischemia reperfusion injury.

However, 1 week following ischemic injury, we observed no reduction in b-wave amplitude relative to the initial baseline readings observed prior to implantation with NTCELL. In fact we observed a moderate increase from immediately prior to ischemia injury induction. Interestingly, the ERG reading of this one animal at 2 weeks post ischemic injury was comparatively similar to that observed in A03 ischemia only rabbit.

The animal was sacrificed at 3 weeks following ischemic injury and capsules retrieved for analysis. Eye tissue was not maintained for histology as it was concluded that the injury was unlikely to have induced an adequate ischemic retinal injury. A total of 47 capsules were retrieved from the approximately 100 capsules which were implanted into the vitreous cavity. Biocompatibility of the capsule appears to be excellent as evident by the absence of any deposition on the surface of the capsule membrane.

In contrast in Figure 7, the viability of the encapsulated choroid plexus clusters do not appear to be as healthy as the clusters retrieved from the capsules during Part 2. A large number of capsules contain clusters which contain living cellular material surrounded by a significant amount dead and/or dying material which presents as loosely packed mass(es). It is reasonable to suggest that the 60 minutes of increased intraocular pressure despite cannulation into the anterior chamber has adversely affected the survival of these clusters in our capsule formulation. However, we do not have an NTCELL without ischemic reperfusion injury model to provide support evidence for this.

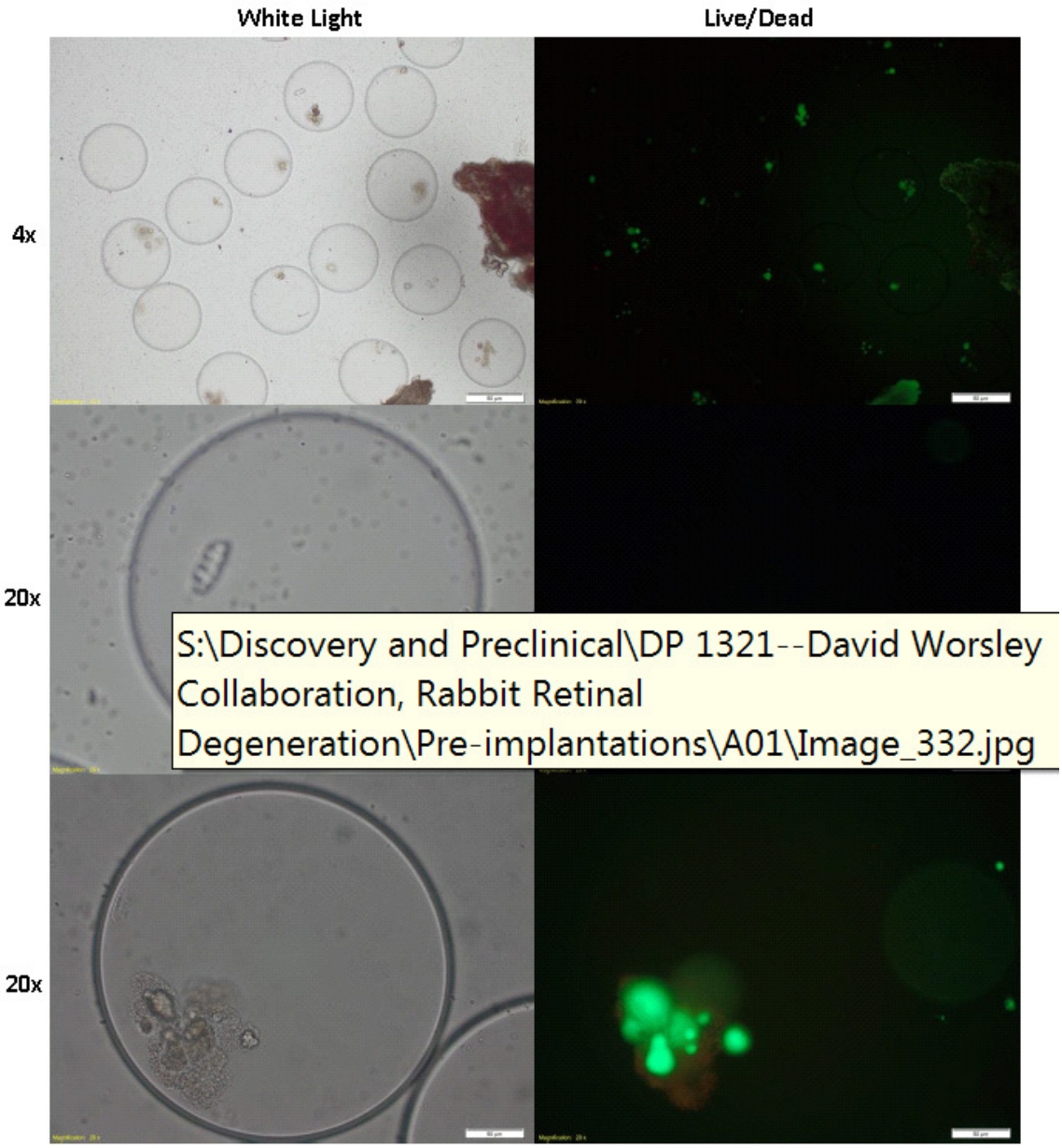


Figure 7. Viability staining with Live/Dead of choroid plexus clusters in capsules retrieved 6 weeks after implantation with NTCELL capsules into the posterior chamber of the left eye of rabbits

A02 – Incomplete ischemia reperfusion procedure, animal was only subjected to 50 minutes of ischemic injury. Animal eliminated from the original pilot study.

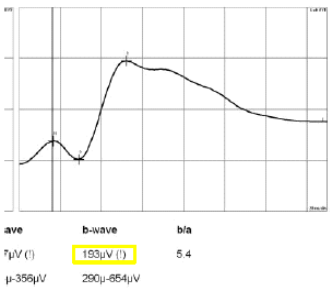


Figure 8. Rabbit A02 ERG profile of the A) Left Eye from baseline prior to 50mins ischemia reperfusion injury

A03 – Successful completion of the ischemia reperfusion procedure however during the first follow up ERG 1 week later showed recovery of the b-wave greater than anticipated. We only observed a 23% reduction in the b-wave compared to the 80-90% as previously described. Furthermore, the ERG measurements at 2 weeks post ischemia injury suggest no further change.

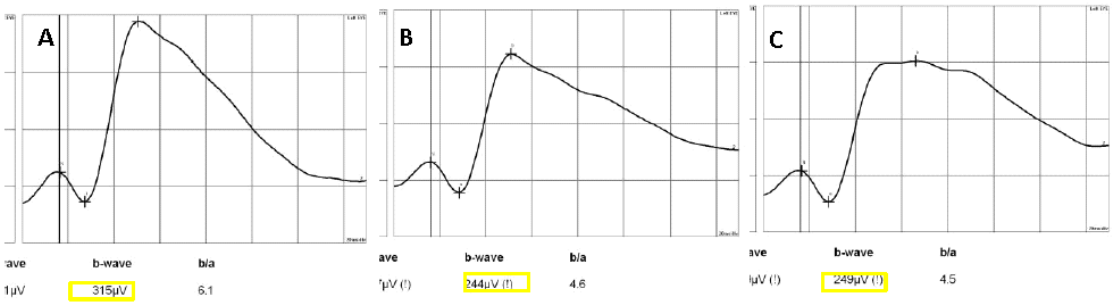


Figure 9. Rabbit A03 ERG profile of the Left Eye from A) baseline prior to ischemia reperfusion injury and B) 1 week and C) 2 weeks following ischemia reperfusion injury

NOTE: For rabbits A02 and A03 there was an observed severe inflammatory response on the surface of the left eye which was not as evident with A01 which had previously received pre-implantations with NTCELL. This was initially thought to be caused by cross contamination of the contact lens used for the ERG measurements from one rabbit to the other. However, when the ischemia injury was repeated in the right eye (DP1321 Part 4) this same inflammatory response occurred. Further enquiries revealed that other investigators using this anterior cannulation approach in rabbits experience an “angry eye” phenomenon and we suggest this is due to a disruption of the aqueous blood barrier of the iris. Interestingly this is not observed in rodent models but occurs frequently and severely in rabbits.

NOTE 2: Use of pain relief/anti-inflammatories was not part of the original application but a visit by a member of the AgResearch Ethics Committee who is also a vet dictated the delivery of a single dose of a NSAID as a requirement for the commencement of the study. This was thought to have first produced a protective effect against retinal injury in A03. However, when this was presented to the committee as a possible influencing factor, and explaining that retina is not innervated with pain fibres, we were able to conduct the injury on the right eye of A02 in DP1321 Part 4 without the use of NSAID. As shown in DP1321 Part 4, removal of the NSAID did not increase the degree of injury observed.

NOTE 3: Xylazine/Ketamine is delivered intramuscularly and intravenously to sedate and anaesthetize rabbits over the duration of the experimental procedures. For increased intraocular pressure induced injury for DP1321 Part 3 (Left eye of A01-A03), the anaesthetist experienced difficulty in retaining the respective animals in a sedated unresponsive state. As a consequence large volumes of xylazine/ketamine were delivered to the animals. Due to sudden movement by A02 the cannula dislodged with release of increased intraocular pressure minutes short of 60mins. This animal had to be removed form the study. It was hypothesized that high doses of these drugs could have also contributed to protecting against a severe injury in A03. It was later discovered that the xylazine had expired and measures have been put into place by the anaesthetist to ensure this is not repeated. Similar to Note 2, standard doses of xylazine/ketamine used in DP1321 Part 4 did not increase severity of ischemic injury.

DISCUSSIONS:

NTCELL group:

· Only 1 animal

· No reduction in b-wave amplitude compared to before ischemia reperfusion injury

· Question: Was the injury successful? Control rabbit ERG's suggest most likely it was not

· No inflammatory response in the days following ischemic injury

· Approximately 50% of capsules were retrieved 6 weeks after the original implantation

· Biocompatibility of capsules was excellent

· Viability of clusters in capsules was variable and possible adversely affected by the increased intraocular pressure exposure

Ischemia Only group:

· Only 1 animal exposed to the entire duration of injury (A03)

· Magnitude of ischemia reperfusion injury was not as severe as initially anticipated

Approximately 30% vs. 80-90%

· A02 (50 minutes ischemia) and A03 (60 minutes ischemia) both experienced an inflammatory response which can be seen on the surface of the left eye

CONCLUSION:

- Retinal ischemia reperfusion injury induced by the increased intraocular pressure method using parameters of 150mmHg for 60minutes is inefficient to produce a reliable model in female NZ White rabbits of approximately 6mths of age.

- Choroid plexus clusters may have been adversely affected by the exposure to increased intraocular pressure

FOLLOW UP STUDY PLAN:

To generate a model which reproducibly induces an injury model which has ERG readouts of 10-20% of baseline at 1 week after injury?

· Using the non-manipulated right eye of rabbits A02, A03 and a new rabbit A04, identify a set of parameters which has the potential to induce consistent reliable ischemia reperfusion injury in rabbits

o 150mmHg 75minutes Anterior Chamber

o 150mmHg 90minutes Anterior Chamber

o 150mmHg 60-90minutes Posterior Chamber

PART 4 DP1321

TITLE: Elucidation of Parameters to obtain a Significant Ischemic Reperfusion Injury in Rabbits using Increased Intraocular Pressure Methods

OBJECTIVE: Establishment of a Reliable Reproducible Ischemic Reperfusion Injury Model through Increased Intraocular Pressure in Rabbits

BACKGROUND/RATIONALE: (see previous study DP1321 Parts 1-3)

DP1321 Part 1: Two male rabbits 22 and 24 weeks respectively responded to increased intraocular pressure (150mmHg 60mins cannulated into the posterior chamber) induced injury (as a model of ischemic reperfusion injury) to produce 80-90% suppression of ERG readouts from baseline after 1 week after injury and were sustained over the 6-8 weeks following. ***Conclusion:*** *It was believed that this model was simple to achieve as 100% of rabbits had responded as expected.*

DP1321 Part 2: Four 15-16 week male and female rabbits did not elicit any significant reduction in ERG readouts from baseline which was first observed at 1 week following injury. ***Conclusion:*** *Younger rabbits are likely to be more resistant to injury induced by increased intraocular pressure to 150mmHg for 60mins. Older rabbits are recommended to obtain the desired injury model.*

DP1321 Part 3: Three rabbits in a pilot study to establish a model for the pre-implantation of NTCELL were unsuccessful. Induction of an increased intraocular pressure model by cannulation of the anterior chamber to minimize the exposure of posterior chamber pre-implanted capsules was only partially successful. ***Conclusion:*** *Increased intraocular pressure model in rabbits needs to be carefully evaluated to obtain the parameters which induce a model which is reproducible and significantly severe for our purposes.*

EXPERIMENTAL DESIGN:

Minimize the number of rabbits used by using the unmanipulated eye of animals which previously were exposed to increased intraocular pressure reperfusion injury but did not induce an injury model which was of adequate magnitude for our purposes (DP1321 Part 3).

Additional animals were requested to the animal ethics committee to complete this part of the study to determine the parameters for sufficient injury.

	Parameter A	Parameter B	Parameter C
Pressure	150mmHg 75mins	150mmHg 120mins	150mmHg 90mins
Chamber	Anterior Chamber	Posterior Chamber	Posterior Chamber
Number of Eyes	1	1	6

Table 6.

RESULTS:

1) Anterior Chamber 150mmHg 75minutes: Successful procedure but did not suppress the b-wave amplitude sufficiently and was considered only partially successful. Also evidence of "angry eye" with observable inflammation of the anterior chamber is likely due to the disruption of the blood-iris aqueous barrier.

2) Posterior Chamber 150mmHg 120minutes: Successful procedure but associated with almost complete ablation of the optic nerve and a flat b-wave response. Injury too severe as we are targeting residue tissue for regeneration.

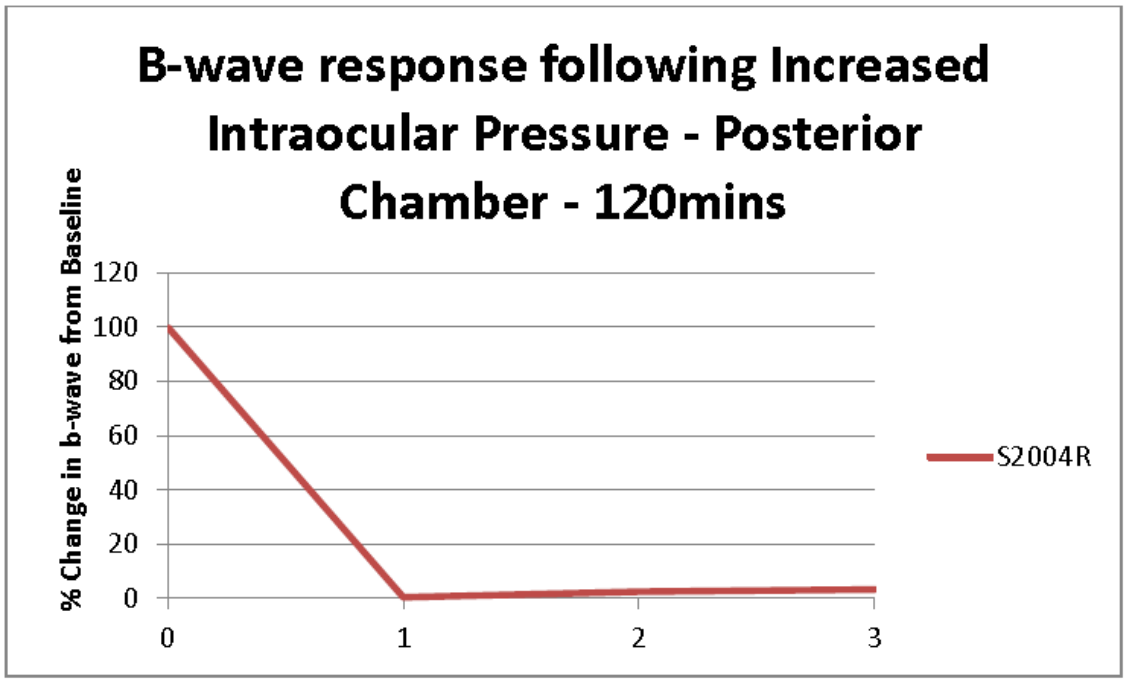


Figure 10.

3). Posterior Chamber 150mmHg 90minutes: One animal with complete ablation of the b-wave almost immediately following the injury but the other five rabbits have moderate suppression of the b-wave amplitude without associated inflammatory response observed when the anterior chamber was cannulated. Greatest injury was observed at 1 week post injury with about 30-40% recovery in the 4 weeks following. After 5 weeks follow up, 40% suppression of the b-wave was still observed. This appears to be the approximate time where recovery has seized.

	Weeks Post Injury (% of baseline b-wave amplitude)					
Rabbit ID	0	1	2	3	4	5
S2004	100	30.28	33.25	50.81	69.11	61.38
S2005	100	24.25	35.50	46.34	64.50	57.99
S2006	100	2.14	4.42	3.22	2.35	5.05
S2007	100	38.77	40.37	50.00	54.55	30.48
S2008	100	42.99	64.02	84.15	81.71	58.54
S2009	100	20.41	32.97	46.26	52.82	31.85

Table 7.

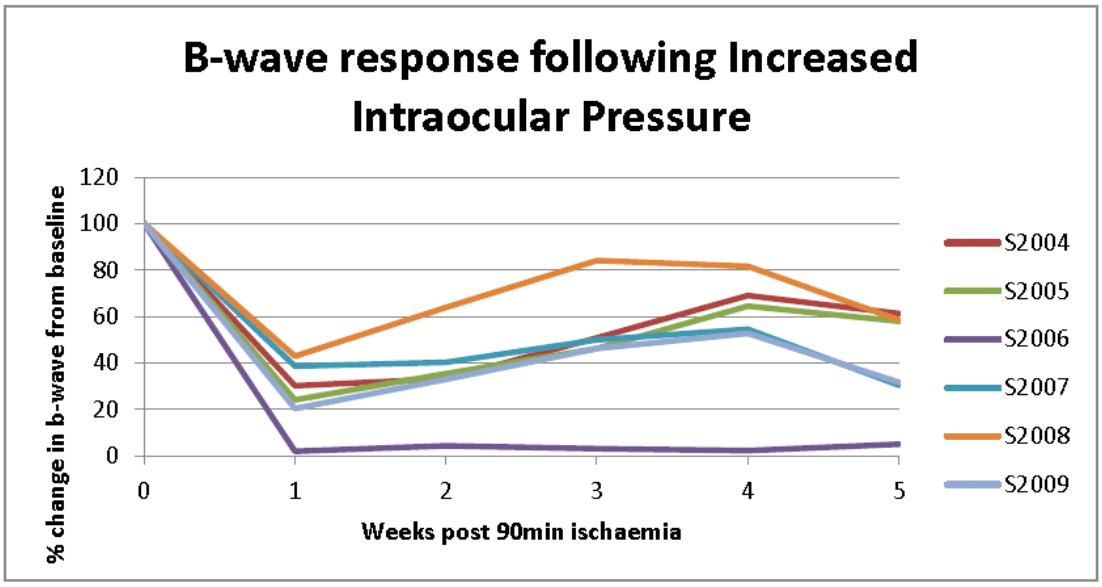


Figure 11.

DISCUSSION:

Maximal injury is observed in the week following 150mmHg 90minutes ischaemic reperfusion injury but moderate recovery is expected in the weeks following. It appears after 5 weeks, this recovery has plateaued. With these parameters we can expect the occasional animal to be severely affected and the result is ablation of the b-wave and blindness but these animals can be immediately eliminated from the study.

NOTE: The “angry eye” which was observed on the left eye of rabbits A02 and A03 (but not A01) was observed on the right eye of rabbit A02 despite measures to eliminate cross contamination (i.e. New lenses used between rabbits). This is apparently common in rabbit eyes cannulated through the anterior chamber but rarely mentioned in publications. It is not seen in rodents.

NOTE 2: Cannulation of the posterior chamber in DP1321 Parts 1 and 2 showed no evidence of inflammatory response. Furthermore, rabbit A04 did not show signs of this effect when 150mmHg 90 minutes into the posterior chamber was used.

CONCLUSION:

· Cannulation of the anterior chamber for 75mins at 150mmHg to increase intraocular pressure as a method of inducing retinal ischemia reperfusion injury in 6mth female rabbits was only partially successful.

· Cannulation of the posterior chamber for 90minutes at 150mmHg to increase intraocular pressure as a method of inducing retinal ischemia reperfusion injury in 6mth female rabbits was successful in suppressing b-wave amplitude response by approximately 70% after 2 weeks. However moderate recovery was observed following 3 weeks after ischemic injury.

· 120mins at 150mmHg cannulated into the posterior chamber induces an injury which results in blindness and damage of the optic nerve. This injury is too severe for our purposes.

- Degree of retinal ischaemic reperfusion injury as a consequence of 150mmHg exposure for 90minutes into the posterior chamber is more variable that first anticipated. This

variability in the model makes it very difficult to interpret any results that are obtained from a treatment group.

- It is recommended that a more stable model of injury be investigated and explored prior to undergoing any treatment testing with NTCELL.

Further Comment

I have revisited the publication used for our method of rabbit retinal ischemia reperfusion. It is by a well respected senior author in a major U.S. institution. However, a review indicates that the results are very likely false. The baseline b waves and the b-wave results of ischemia reperfusion are extra-ordinarily reliable. In every one of 24 animals the baseline and depression of b wave following ischemia (of a degree ineffectual in our rabbits) is very close with a range of within 10-20mv. In each group with a different manipulation the resulting b wave is again very close in all animals. Statistically, this is very unlikely to have occurred. We looked at the baseline ERG in both eyes of some of our rabbits (same sex siblings of same age and raised together). Between eyes in a healthy rabbit the b-wave ERG can vary by 20-30% confirmed by repeating. Between animals the variability is even greater. In normal humans scotopic b-wave has a 25% variance from one to the other eye and the same eye can vary by a similar percentage on different days. Data manipulation is common in animal research to secure publication. We may have fallen into the trap of believing published work to be correct and thereby have chosen an unreliable model. Interestingly, rabbit retinal models of light induced damage are very unreliable yet are very reliable in rodents. Recent enquiries I have made in the USA suggest that all rabbit retinal models should be used cautiously. Unfortunately, this was unknown to me before despite talking to several experts before commencing the project. There is not a huge rabbit experience; nearly all workers have used rodents as they have not needed bigger eyes. Dogs are commonly used for bigger eyes but this was not feasible for us. These negative opinions on rabbit models are not in the literature.

Future work for Proof of Principle of NTCELL for retinal degeneration

I am now doing preliminary work on the rat eye. This will require the removal of the lens which occupies about 95%+ of the eye volume. Otherwise the vitreous space is about 0.2 ml and not a reproducibly feasible eye for implantation. I did manage to implant capsules in a few rat eyes prior to the rabbit work but dense cataract resulted in the vast majority and a few eyes became disorganized. Only a very small number were successful.

Initial experience indicates that removal of the lens is technically easy but is a major insult to the eye in the short term. There is always the possibility of a problem using a model with an eye without a lens (aphakic). Physiologically an aphakic eye is significantly different from a normal eye. There is now communication between anterior and posterior segments and, for example, the secretome from NTCELL possibly may be rapidly removed from the eye via the anterior segment.

Should the aphakic rat eye have healthy functional retina then high intraocular pressure retinal ischemia reperfusion is a well validated model. Professor Colin Green, Auckland Medical School, and also the optometry school have used this reliably. My plan is to use the model at the Auckland Medical School and have input from the workers with prior experience.