

**The Development of Human Organotypic Retinal Cultures (HORCs) to study  
Retinal Neurodegeneration**

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Competing Interest: None to declare

## **Abstract**

**Aims** To develop human organotypic retinal cultures (HORCs) to study retinal ganglion cell (RGC) death in response to ischaemic and excitotoxic insults, both known to cause loss of RGCs and proposed as mechanisms involved in glaucomatous retinal neurodegeneration.

**Methods** Human donor eyes were obtained within 24hr *post mortem*. The retina was isolated and explants cultured using two techniques. *THY-1* mRNA (assessed by real time quantitative PCR) and neuronal nuclei (NeuN) (assessed by immunohistochemistry) were used as markers of RGCs. Apoptosis was assessed by TUNEL labelling.

**Results** The distribution of *THY-1* mRNA and NeuN-labelling within the human retina was consistent with expected distribution of RGCs. Gross morphology and retinal architecture remained stable over a 96hr culture period. *THY-1* mRNA and NeuN-labelled RGC layer cells decreased over the culture period and there was an increase in TUNEL-labelling with time, but HORCs cultured in serum-free DMEM/HamF12 medium were useful for up to 48hr in culture. NMDA (10µM) caused a reduction in *THY-1* mRNA by 24hr and decreased the numbers of NeuN-labelled RGC layer neurones by 48hr, suggesting that the loss of *THY-1* mRNA was a marker of RGC stress prior to death. Simulated ischaemia (60 minute oxygen/glucose deprivation) caused a reduction at 24hr in both *THY-1* mRNA and the numbers of NeuN-labelled neurones of HORCs.

**Conclusion** HORCs provide a useful model to investigate RGC insult by neurodegenerative mechanisms that may lead to glaucoma in human eyes.

## **Introduction**

Organotypic explant cultures allow controlled experimentation of retinal tissue and have the advantage over dissociated cultures by retaining regional morphology. Animal organotypic retinal cultures (ORCs) have been developed to investigate retinal physiology [1-3]. Adult rabbit ORCs retained morphology and electrophysiological function for  $\leq 6$  days, suggesting intact neural circuitry [3]. Furthermore, viability in rat ORCs has been demonstrated for  $\geq 17$  days [4] making them useful for neuroprotection studies. For example, rat [5] and mouse [6] ORCs have been used to investigate axotomy-induced RGC death and the neuroprotective effect of caspase inhibitors. Glutamate and *N*-methyl-D-aspartate (NMDA) excitotoxicity of RGC layer cells and the neuroprotective effect of NMDA antagonists have also been studied in both rat [7] and rabbit [8] ORCs.

We aimed to develop human organotypic retinal cultures (HORCs) to investigate loss of RGCs induced by either excitotoxicity or ischaemia, insults known to cause RGC death in *in vivo* and *in vitro* models of glaucoma [9, 10].

## **Materials and Methods**

### **Human Organotypic Retinal Cultures (HORCs)**

Donor human eyes were obtained within 24hr *post mortem* (PM) from the East Anglian Eye Bank with full ethical approval, under the tenets of the Declaration of Helsinki. Eyes with known pathology were excluded. Under sterile conditions, the neural retina was detached from the retinal pigment epithelium and choroid, dissected at the optic nerve and transferred RGC-side up into CO<sub>2</sub>-independent medium (Gibco

Invitrogen, UK). The retinal edges were cut, allowing the retina to lie flat. 4mm diameter micro-dissecting trephine (Biomedical Research Instruments, USA) punch retinal explants were placed into serum-free Dulbecco's Modified Eagle Medium (DMEM)/HamF12 (Gibco Invitrogen, UK) in 35mm dishes (Corning, USA), or onto Millicell inserts (12mm diameter; 0.4µm pore) (Millipore, UK) RGC-side up in Neurobasal A, supplemented with B27/N2 (Gibco Invitrogen, UK) in a 12-well plate (NUNC, Denmark). Both media contained 50µg/ml gentamicin (Sigma-Aldrich, UK). Each HORC was placed into a separate culture dish or well. HORCs were incubated at 37°C in a humidified atmosphere of 95%O<sub>2</sub>/5% CO<sub>2</sub>. The medium was unchanged during the course of experiments unless necessitated by protocol (see oxygen/glucose deprivation below).

### **Neurotoxic insults**

#### ***N*-methyl-D-aspartate excitotoxicity**

*N*-methyl-D-aspartate (10µM) (Tocris Bioscience, UK) was added to the HORCs in serum-free DMEM/HamF12 .

#### **Oxygen/glucose deprivation (OGD) (simulated ischaemia)**

HORCs were placed in serum-free DMEM without glucose and pyruvate (Gibco Invitrogen, UK), previously warmed and bubbled with 95%N<sub>2</sub>/5%CO<sub>2</sub> for 10min to remove oxygen. HORCs were placed in a modular incubator container (Billups-Rothenburg, USA) and gassed (10min) with humidified 95%N<sub>2</sub>/5% CO<sub>2</sub> before sealing the chamber. HORCs were incubated for a further 50min, then switched to serum-free DMEM/HamF12 and incubated for 24hr. Control HORCs were placed into DMEM with glucose (Gibco Invitrogen, UK) for the initial 60min incubation period.

### ***THY-1* mRNA expression by Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)**

Total RNA was extracted from HORCs using the RNeasy Mini Kit column-based method (Qiagen, UK). The concentration of total RNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Total RNA was reverse transcribed to synthesise cDNA using a Peltier Thermal Cycler DNA engine (PTC-200, MJ Research, USA). The ABI Prism 7700 Sequence Detection System (Applied Biosystems, UK) was used for QRT-PCR. cDNA was mixed with Human *THY-1* probe (Hs00174816\_m1, Applied Biosystems, UK) and TaqMan® PCR Master Mix (Applied Biosystems, UK).

The geNorm programme (<http://medgen.ugent.be/~jvdesomp/genorm>) identified the most stable house-keeping genes for HORCs as cytochrome c-1 (*CYCI*), topoisomerase DNA I (*TOPI*) and ATP synthase H<sup>+</sup> transporting mitochondrial F1 complex  $\beta$  subunit (*ATP5B*). The geometric mean of C<sub>T</sub> values of either *CYCI* and *TOPI* or *TOPI* and *ATP5B* were used as described previously [11] to normalise the data for *THY-1* mRNA.

### **Immunohistochemistry**

HORCs were fixed in 4% paraformaldehyde for 24hr and cryopreserved in 30% sucrose in phosphate buffered saline (PBS) (Oxoid, UK) for a further 24hr, before being placed into cryostat block moulds filled with optimal cutting temperature medium (Sakura Finetek, Netherlands) and frozen on dry ice. Retinal slices (13 $\mu$ m)

were cut using a Hacker Bright OTF 5040 cryostat (Bright Instruments, UK) and collected on 3'-aminopropyl-triethoxyl silane (TESPA)-coated glass slides.

Retinal slices were blocked in 5% normal goat serum (Sigma-Aldrich, UK) with 0.2% Triton X-100 (Sigma-Aldrich, UK) in PBS for 90min (room temperature) before incubating with primary antibody (1:200) for mouse neuronal nuclei (NeuN) (Millipore, UK) at 4°C overnight. Secondary antibody (goat anti-mouse AlexaFluor 488; Invitrogen, UK) was used at 1:1000 and incubated for 2hr (room temperature). The nuclei were counterstained with 4',6-diamidino-2-phenylindole diacetate (DAPI) (1:100) for 10min (room temperature). Slides were mounted in immunofluorescence mounting medium (National Diagnostics, UK). A wide-field Zeiss Axiovert 200M fluorescent microscope (Zeiss, UK) was used with Zeiss Axiovision 4.7 software.

NeuN is a well-characterised RGC marker [12, 13] and NeuN-labelling in HORCs was co-localised with nuclei in the RGC layer (Supplementary Figure 1). The number of NeuN-labelled RGC layer cells was used to determine RGC number and inversely as an index of RGC death. Six non-consecutive immunohistochemical slices were prepared for each HORC and NeuN-stained RGC layer cells counted (masked) in three 200µm sections. The mean for 6 slices was taken as one biological replicate.

### **TUNEL Assay**

Apoptosis in the HORCs was detected by TUNEL assay (Promega Life Sciences, UK). TUNEL staining was carried out in conjunction with NeuN immunohistochemistry. Following incubation with primary antibody, slides were incubated with TUNEL reagent for 1hr at 37°C. The reaction was stopped using

standard citrate solution prior to addition of secondary antibody. Slides were then processed and analysed as described above.

### **Statistical Analysis**

Significance was analysed with a non-paired Student's t-test. A probability lower than 5% was considered statistically significant.

### **Results**

#### **Human retinal *THY-1* mRNA distribution reflected expected RGC distribution**

Explants were taken from macular, para-macular and peripheral retinal regions at the time of dissection (Figure 1A) and assessed for *THY-1* mRNA. Macular *THY-1* mRNA was highest, at greater than twice the level for the para-macular region, the latter having approximately twice the level compared with peripheral regions (n=4 donor eyes; Figure 1B), reflecting the expected retinal distribution of RGCs. Labelling of RGCs using NeuN immunohistochemistry also followed the same distribution (Figure 1C-E). There was no significant difference between the amount of *THY-1* mRNA in retinal explants from the 5 para-macular regions (n=6 donor eyes; Figure 2A) and numbers of NeuN-labelled RGC layer neurones were found to be similar in each (n= 4 donor eyes; Figure 2B). Due to the similar levels of *THY-1* mRNA and NeuN-stained RGC layer neurones, the 5 para-macular regions from each eye were used for development of HORCs to study RGC death. Retinas were not used beyond 24hr *PM*, since *THY-1* mRNA levels tended to be low thereafter. Within



24hr, no correlation between *THY-1* mRNA levels in the para-macular regions and *PM* time was identified.

### **Optimal culture medium for HORCs**

Previous studies have used a variety of media and culture conditions for ORCs [3, 4, 14-16]. In the present study, the viability of HORCs was compared under two culture systems: placing the HORCs directly into serum-free DMEM/HamF12 and culture on inserts (RGC-side up) in Neurobasal A medium plus B27/N2 supplements.

Over 96hr no difference in gross appearance (architecture, opacity and colour) of HORCs was identified between culture conditions. There was a similar reduction in *THY-1* mRNA levels in both conditions, with an approximate 50% decrease at 24hr and a further 25% decrease at 48hr, reaching basal levels after 72hr (n=4 donor eyes; Figure 3A). Subsequent experiments were performed in serum-free DMEM/HamF12 since medium composition was known. Over the 96hr period, gross appearance remained unchanged (Figure 3B). Retinal architecture remained intact over the culture period with no notable decrease in thickness/density of the nuclear layers (Figure 3C-G). NeuN-labelled cell number remained stable over the first 48hrs, but decreased at the 72 and 96hr time points (Figure 3C-H). The numbers of TUNEL-positive apoptotic nuclei were also assessed (Figure 3C-G). In freshly dissected retina no TUNEL-positive nuclei were observed (Figure 3C). At 24 and 48hrs apoptotic cells were observed mainly in the inner nuclear layer (Figure 3D&E). By 96hrs some TUNEL-positive cells were apparent in all nuclear layers, including co-localisation between NeuN- and TUNEL-staining (Figure 3G arrows).

### ***N*-methyl-D-aspartate excitotoxicity in HORCs**

*N*-methyl-D-aspartate (10 $\mu$ M) caused a significant reduction (19 $\pm$ 8%) in *THY-1* mRNA expression in HORCs compared to controls at 24hr ( $p < 0.05$ ,  $n = 4$  donor eyes; Figure 4A). At the equivalent time point, there was no difference in number of NeuN-labelled RGC layer cells between control (mean 8 $\pm$ 3 NeuN-labelled neurones/200 $\mu$ m) and NMDA-treated HORCs (mean 7 $\pm$ 1 NeuN-labelled neurones/200 $\mu$ m) ( $n = 4$  donor eyes; Figure 4B, E & F). However, by the 48hr time point a significant reduction of 50 $\pm$ 6% was observed ( $p < 0.05$ ,  $n = 4$  donor eyes; Figure 4C, G & H).

### **Oxygen/glucose deprivation (OGD) (simulated ischaemia) in HORCs**

Oxygen/glucose deprivation for 60min caused a significant reduction (44 $\pm$ 13%) in *THY-1* mRNA expression at 24hr compared to controls ( $p < 0.05$ ,  $n = 4$  donor eyes; Figure 5A). OGD (60 min) also caused a significant reduction (40 $\pm$ 8%) in NeuN-labelled RGC layer neurones at 24hr compared to controls ( $p < 0.05$ ,  $n = 5$  donor eyes; Figure 5B-E).

### **Discussion**

We have developed a simple system for culture of human retinal explants to investigate RGC death. Our specific interest in RGC cells led us to investigate the distribution of RGC markers in the human retina for the purpose of selecting comparable regions for subsequent use in HORC-based studies. *THY-1* mRNA distribution reflected the known retinal RGC density distribution and was at a similar level in 5 para-macular regions. Thy-1 is almost uniquely expressed by RGCs [17] and has been used as an index of RGC death in ischaemic and excitotoxic retinal injury [10]. NeuN is another marker of RGCs, giving extensive labelling in the RGC

layer, whilst labelling only a minority of inner nuclear layer cells and none of the photoreceptor layer [12] - this was consistent with our findings. For experimentation, the 5 para-macular regions from each donor retina were selected for HORCs since they contained similar numbers of NeuN-stained RGC layer neurones and useful amounts of *THY-1* mRNA. Most eyes were dissected and cultured within 12hr *PM*, although human retinas were still useful within 24hr.

A number of studies have developed animal ORCs in DMEM/HamF12 culture medium with the addition of various supplements including serum [8, 14, 18, 19]. Others have found that Neurobasal culture medium with supplements optimised ORC viability [4, 5, 20, 21]. The simplest method of culturing ORCs involved placing the explants directly into medium, which maintained the ORCs for at least 5 days [15, 16, 22]. The optimal medium for rat ORCs was determined to be serum-free Neurobasal A medium supplemented with B27/N2, with explants supported on cell culture inserts, RGC-side up [4]. These conditions maintained explant viability for  $\geq 17$  days compared to explants in media containing normal horse serum that started to degenerate at 10 days *in vitro* [4]. In order to determine suitable culturing conditions for HORCs, this method was compared to the simplest method of placing the HORCs directly into serum-free DMEM/HamF12. Experiments indicated that the viability of HORCs in serum-free DMEM/HamF12 and serum-free/nutrient-supplemented Neurobasal A medium was equal, hence DMEM/HamF12 medium was selected since the exact medium composition was known. Excessive manipulation of HORCs was found to be detrimental and because placing the HORCs directly into medium was less traumatic than manipulating the tissue onto culture inserts, the former method was utilised. The surface tension of the medium helped flatten the HORCs avoiding

the need for further ‘trauma’. The HORCs floated just below the surface of the medium, which may have allowed advantageous gaseous exchange.

Freshly dissected human retina showed robust *THY-1* mRNA expression, NeuN-labelling and, importantly, no evidence of apoptosis. Consistent with data from explants derived from adult rat [6], this indicated a lack of significant apoptotic events during the *PM* period. With culture duration, there was a reduction in *THY-1* mRNA and the number of NeuN-labelled RGC layer neurones in the HORCs. The rate of loss of *THY-1* mRNA was greater than that for NeuN-labelling; at the 48hr time point basal *THY-1* expression had been reached, but there was no significant loss of NeuN-labelling and no increase in TUNEL-labelling in the RGC layer. This indicates that the level of *THY-1* mRNA was a more sensitive index of RGC health. Time-dependent loss of cultured RGCs has not previously been determined using HORCs, but has been shown to occur in animal ORC models, although at a slower rate [5, 6, 23].

By using our HORC methodology there were still useful amounts of *THY-1* mRNA for  $\leq 36$ hr in culture and useful numbers of NeuN-labelled RGC layer neurones for  $\leq 48$ hr. The best time-point to assess *THY-1* mRNA in HORCs subjected to neurotoxic insults was 24hr, when there was a significant reduction in *THY-1* mRNA following exposure to 10 $\mu$ M NMDA and 60min simulated ischaemia. The reduction in *THY-1* mRNA preceded loss of NeuN-stained neurones from the RGC layer following NMDA excitotoxicity. At 24hr, there was no difference between NeuN-labelled RGC layer neuronal cell counts in control and NMDA-treated HORCs, although at 48hr there was a 50% reduction. The reduction of *THY-1* mRNA prior to the loss of RGCs

has been reported previously in rodent eyes subjected to optic nerve crush, intravitreal NMDA injection or elevated intraocular pressure [24]. Consistent with the time course data discussed above, this suggests that the reduction in *THY-1* mRNA appears to be a marker of RGC stress before cell death. Using HORCs, we have shown that NMDA excitotoxicity and simulated ischaemia caused a reduction of RGC markers in the human retina. HORCs, therefore, provide a useful model to study RGC insult by neurodegenerative mechanisms that may lead to glaucoma.

*In vivo* models are 'ideal' for studying glaucomatous processes; however, they lack *in vitro* model versatility that allows changing experimental conditions. Primate models are more relevant to human glaucoma than rat and mouse models [25]. However, the lack of experimental primate availability and high costs limit their use for investigating glaucoma. HORCs provide a model for investigating the processes involved in RGC death and human retinal neuroprotection, with greater relevance to the clinician. In addition, retinal explant models are valuable for investigating the processes involved in RGC axonal regeneration [26-29]. By using different substrates HORCs may also be useful to study axon regrowth; this certainly warrants further investigation.

There are some limitations with ORCs. Unlike hippocampal slices that can be maintained for weeks, retinal neurones in explant cultures can be difficult to maintain for long periods, perhaps due to the high energy requirements of the retina [30]. The development of ORCs involves transection of the optic nerve and retrograde RGC degeneration. Recognising that there is a loss of RGCs with time in these cultures is important when interpreting results and thus adequately time-controlled experiments

are essential. In the present study, the use of similar para-macular regions from each donor made the use of HORCs ideal for controlled experimentation.

The main problem with HORCs is that completely fresh human tissue cannot be obtained (cf animal tissue). The relatively long *PM* interval means that processes involved in cell death are likely to be already activated. However, it should be noted that in the freshly processed samples TUNEL-labelling was not observed. An additional reason for the relatively poor survival of cultured human retina could relate to differences in vascular anatomy between animals and humans. Specifically, the relative avascularity of the rabbit retina may explain the success of detecting RGC electrophysiological recordings from rabbit retinas for  $\leq 6$  days in culture [3], since rabbit retina may extract nutrients from medium in a similar way to that from the choroid *in vivo*. In the human retina, branches of the central retinal vessels, supply the inner two-thirds of the tissue with only the outer third relying on choroidal nourishment. It is possible that the human retina may be unable to achieve the same supply of nutrients from culture medium as rabbit retina.

In summary we have developed a human retinal explant culture model that can be used to investigate RGC death from neurotoxic insults such as NMDA excitotoxicity or ischaemia. HORCs are useful for studying the effects of neurotoxic insults for  $\leq 48$ hr. HORCs may represent a good model to investigate the processes that lead to RGC death and neuroprotection in human glaucoma.

## Figure legends

Supplementary Figure 1. Immunofluorescence photomicrograph of the retinal ganglion cell layer of a freshly dissected human retina showing co-localisation of NeuN (green) and the nuclear stain DAPI (blue). GCL, ganglion cell layer; INL, inner nuclear layer.

Figure 1. (A) Freshly dissected human retina within 24hr *post mortem* showing the areas of sampling from the central macular (red), para-macular (blue) and peripheral (yellow) regions of the retina. (B) Normalised *THY-1* mRNA (mean  $\pm$  S.E.M.) of central macular retinal explants (red), para-macular retinal explants (blue) and peripheral retinal explants (yellow) at time 0hr (n=4 donor eyes). *THY-1* mRNA expression was normalised relative to the expression of *TOPI* and *ATP5B* mRNA. Representative immunofluorescence photomicrographs of regions of freshly dissected human retina (C) central macular, (D) para-macular and (E) retinal periphery stained with NeuN (green) and the nuclear stain DAPI (blue) showing a decrease in NeuN-labelled retinal ganglion cell layer neurones with distance from the central macular. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

Figure 2. (A) Normalised *THY-1* mRNA level (mean  $\pm$  S.E.M.) in the 5 para-macular regions of the retina at time 0hr (n=6 donor eyes). *THY-1* mRNA expression was normalised relative to the expression of *TOPI* and *ATP5B* mRNA. (B) 200 $\mu$ m NeuN-labelled neuronal cell counts (mean  $\pm$  S.E.M.) of the retinal ganglion cell layer of the 5 para-macular regions (n=4 donor eyes).

Figure 3. (A) Normalised *THY-1* mRNA (mean  $\pm$  S.E.M.) from HORCs in culture in serum-free DMEM/HamF12 (blue) and HORCs placed on Millicell culture inserts with serum-free Neurobasal A medium and B27/N2 supplements (red) at 0, 24, 48, 72 and 96 hrs (n=4 donor eyes). *THY-1* mRNA expression was normalised relative to the expression of *TOP1* and *CYCI* mRNA. (B) Photomicrographs of HORCs in culture at 0, 24, 48, 72 and 96 hrs, showing no change in the gross appearance with time in culture. Representative immunofluorescence photomicrographs of HORCs after (C) 0 hrs, (D) 24 hrs, (E) 48 hrs, (F) 72 hrs and (G) 96 hrs in culture, stained with TUNEL (red), NeuN (green) and the nuclear stain DAPI (blue), showing an intact retinal architecture over the culture period and an increase in TUNEL staining particularly in the inner nuclear (INL) and outer nuclear (ONL) layers with time in culture, but also in the retinal ganglion cell layer (GCL) at 96 hrs. Arrows indicate co-localised NeuN- and TUNEL-stained retinal ganglion cell layer neurones. (H) 200 $\mu$ m NeuN-labelled neuronal cell counts (mean  $\pm$  S.E.M.) of HORCs after 0, 24 and 48 hrs in culture (n=4 donor eyes).

Figure 4. (A) Normalised *THY-1* mRNA (mean  $\pm$  S.E.M.) of control and 10 $\mu$ M NMDA-treated HORCs at 24hr expressed as a percentage of 24hr control *THY-1* mRNA (\* p<0.05, n=4 donor eyes). *THY-1* mRNA expression was normalised relative to the expression of *TOP1* and *CYCI* mRNA. (B) 200 $\mu$ m NeuN-labelled neuronal cell counts (mean  $\pm$  S.E.M.) of control and 10 $\mu$ M NMDA-treated HORCs at 24hr expressed as a percentage of 24hr control (n=4 donor eyes). (C) 200 $\mu$ m NeuN-labelled neuronal cell counts (mean  $\pm$  S.E.M.) of control and 10 $\mu$ M NMDA-treated HORCs at 48hr expressed as a percentage of 48hr control (\* p<0.05, n=4 donor eyes).



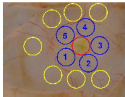
Representative immunofluorescence photomicrographs of (D) 0hr control, (E) 24hr control, (F) 24hr 10 $\mu$ M NMDA-treated, (G) 48hr control and (H) 48hr 10 $\mu$ M NMDA-treated HORCs stained with NeuN (green) and the nuclear stain DAPI (blue) showing no difference in the number of NeuN-labelled neurones between control and 10 $\mu$ M NMDA-treated HORCs at 24hr, but a reduction in NeuN-labelled neurones in the retinal ganglion cell layer (GCL) of 10 $\mu$ M NMDA-treated HORCs compared to controls at 48hr. INL, inner nuclear layer; ONL, outer nuclear layer. Statistical analysis: Student's T-test.

Figure 5. (A) Normalised *THY-1* mRNA (mean  $\pm$  S.E.M.) of control and 60min oxygen/glucose deprivation (OGD) HORCs at 24hr expressed as a percentage of 24hr control (\*  $p < 0.05$ ,  $n = 4$  donor eyes). (B) 200 $\mu$ m NeuN-labelled neuronal cell counts (mean  $\pm$  S.E.M) of control and 60min OGD HORCs at 24hr expressed as a percentage of 24hr control (\*  $p < 0.05$ ,  $n = 5$  donor eyes). Representative immunofluorescence photomicrographs of control HORCs at (C) 0hr and (D) 24hr in culture and (E) 60min OGD HORN after 24hr in culture stained with NeuN (green) and the nuclear stain DAPI (blue) showing a reduction in NeuN-labelled neurones in the retinal ganglion cell layer (GCL) of 60min OGD HORCs compared to controls at 24hr. INL, inner nuclear layer; ONL, outer nuclear layer. Statistical analysis: Student's T-test.

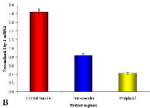
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**A**

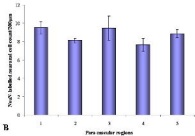
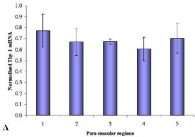


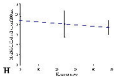
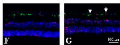
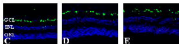
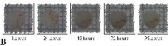
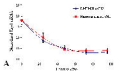
**B**



**D**

**E**







0 hours



24 hours



48 hours



Control

200µM H<sub>2</sub>O<sub>2</sub>

100µm