It's About Time: Time-Dependent Tissue Damage in the Adult Porcine Retina After Enucleation

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Keywords

Porcine retina · Large animal model · Enucleation · Apoptosis · Culture

Abstract

The ex vivo large animal retina is extensively used in research ranging from discovery of disease mechanisms to future treatment paradigms. Due to limited standardization when harvesting the tissue, the time after enucleation is often extended for several hours, a factor that so far has not yet been fully characterized. The purpose of this study was to investigate the relationship between time after enucleation and retinal tissue damage. Adult, porcine retinal explants were dissected and fixed 90 or 240 min after enucleation. In a separate experiment, explants were cultured for 48 h, following dissection either 90 or 240 min after enucleation. Retinas were analyzed morphologically using hematoxylin and eosin for overall tissue damage, TUNEL staining for detection of apoptosis, and RBPMS immunohistochemistry for evaluation of ganglion cell survival. In addition, medium from the cultured explants was sampled after 2, 24, and 48 h of culture and assessed for the cell damage marker lactate dehydrogenase (LDH). Retinas examined 240 min after enucleation displayed a significant increase in overall tissue damage, increased apoptosis, and decreased ganglion cell survival

compared with 90-min counterparts. In the culture experiment, no significant difference in overall tissue damage was found between the 2 groups, however, apoptosis was significantly increased, and ganglion cell survival decreased in the cultured 240-min group. In addition, a significantly increased LDH medium activity was found in the 240-min group compared with the 90-min counterpart at all time points. The adult porcine retina is relatively resistant to tissue damage 90 min after enucleation but displays distinct signs of injury after 240 min. The importance of these time points is further highlighted when retinal explants are cultured. Our results strongly suggest that time after enucleation is a crucial factor that should be considered in experiments involving the ex vivo adult porcine retina.

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Introduction

The isolated adult retina is extensively used in contemporary research for explorations of pathological disease mechanisms and tissue engineering, as well as in treatment paradigms ranging from pharmacological intervention to experimental retinal transplantation [for a review, see Rettinger and Wang, 2018]. Most experiments have explored retinal tissue from small animals, but research

involving large animal models, including porcine and bovine eyes, has gained interest in recent years due to an enhanced similarity to the human retina.

When working with large animal models, the tissue used for retinal experiments is frequently harvested from local abattoirs, which limits the possibility of a standardized handling process, and the time after enucleation is often extended for several hours [Schnichels et al., 2019]. The retina degenerates postmortem after enucleation due to global ischemia as well as axotomy of ganglion cell axons. To limit tissue damage, enucleated eyes can be kept under hypothermic conditions, which in a few studies has been shown to, at least partly, attenuate ganglion cell death [Reinhard et al., 2016; Schultheiss et al., 2016]. However, the influence of time from enucleation on overall retinal tissue damage, as well as any impact on subsequent investigations in the large animal model eye, has not yet been thoroughly explored.

For the present study, we wanted to expand the knowledge on time after enucleation as a relevant factor for experimental research involving the adult retina in a large animal model, with the aim of systematic characterization of time-dependent tissue damage per se and exploration of any extended effects in vitro. Previous in vivo and clinical investigations have indicated that the retinal tolerance time to ischemia is approximately 90 min and that extensive tissue destruction is evident after 240 min [Hayreh et al., 2004]. These time points were therefore chosen for the present experiment in which we investigated retinal tissue damage in porcine eyes 90 or 240 min after enucleation, with the addition of experiments in which we cultured retinal explants after these time points to explore whether an increased time after enucleation has an extended impact in vitro.

Materials and Methods

Animals

Adult pigs aged between 4 and 6 months were transported from a local breeder to the Biomedical Center at Lund University where all experiments were performed. Animals were sacrificed after sedation by an intravenous overdose of sodium pentobarbital (Apoteket, Umeå, Sweden), after which both eyes were enucleated and placed in a vial containing CO₂-independent culture medium (Invitrogen, Paisley, UK) at 4°C on ice. Eyes were immediately transported to the laboratory and dissected at room temperature 90 or 240 min after enucleation as follows. The anterior segment was removed by sharp incision in the pars plana 360°, and the vitreous carefully removed using sterilized tissue paper. The neuroretinas were gently dissected free from the pigment epithelium with micro-forceps, and the optic nerve was cut using micro-scissors. Six full-thickness retinal pieces measuring approximately 5 × 5 mm

were dissected from the centrally located area on the temporal and nasal side of the optic nerve head between the 3 major vascular arcades as previously described [Taylor et al., 2013]. After dissection, retinal pieces were either immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 for 2 h at room temperature, or cultured as described below.

For exploration of time-dependent tissue damage after enucleation, retinal pieces were immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, 90 or 240 min (±10 min) after enucleation (*n* = 12 in each group).

For the culture experiment, additional pieces from each time point (*n* = 12 in each group) were explanted onto Millicell-PCF 0.4 mm culture plate inserts (Millipore, Billerica, MA, USA), with the inner retina facing the membrane. This orientation of explants was chosen to optimize tissue survival in vitro [Taylor et al., 2014]. The 24 retinal explants were cultured in Dulbecco's modified Eagle's medium F12 (DMEM/F12; Invitrogen). The medium was supplemented with 10% fetal calf serum (Sigma-Aldrich, St Louis, MA, USA) and 1% antibiotics (2 mM L-glutamine, 100 U/mL penicillin, and 100 ng/mL streptomycin; Sigma Aldrich). Explants were maintained at 37°C (95% humidity and 5% CO₂ + 95% air) for 48 h after which they were fixed as described above. The culture time points were chosen based on previous experiments in which the tissue was relatively well preserved [Åkerström et al., 2017].

Histology

After several rinses in phosphate buffered saline (PBS), the fixed tissue explants were infiltrated with 0.1 M Sörensen's solution with increasing concentrations of sucrose up to 25%. They were then embedded in egg albumin/gelatine medium, cryosectioned at 12 µm, and transferred to glass slides. For light microscopy, slide 1, 10, 20, and 25 were stained with hematoxylin and eosin (H&E). For immunohistochemical labeling of ganglion cells, slides were rinsed 3 times with PBS and then incubated with PBS buffer containing 0.25% Triton-X and 1% bovine serum albumin (BSA), for 20 min at room temperature. The specimens were then incubated overnight at 4°C with an antibody raised against RNA-binding protein with multiple splicing (RBPMS; PhosphoSolutions, Aurora, CO, USA), a specific ganglion cell marker [Rodriguez et al., 2014]. The specimens were then rinsed in PBS and incubated for 45 min with a rhodamine red-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA), and finally mounted in Vectashield mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA, USA). Negative control experiments were performed as above, replacing the primary antibody with PBS containing 0.25% Triton-X and 1% BSA.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using the TMR red In Situ Cell Death Detection kit supplied by Roche Diagnostics GmbH, Mannheim, Germany.

Lactate Dehydrogenase Assay of Medium from Cultured Explants

For assessment of overall cell viability, 240 µL of medium derived from retinal explants cultured 90 and 240 min after enucleation, was sampled after 2, 24, and 48 h and stored at -80°C. After thawing, the samples were diluted (1:3) with PBS and analyzed for lactate dehydrogenase (LDH) activity using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA), according to the instructions from the manufacturers.

Table 1. Tissue damage score for quantification of the morphology in the inner and outer retina in H&E-stained retinal explants

	Score	Inner retina	Outer retina
Lamination	0	Normal organization of cell bodies in the INL	Normal organization of cell bodies in the ONL
	1	Mild displacement and/or disorganization of cell bodies in the INL	Mild displacement and/or disorganization of photoreceptor cell bodies in the ONL
	2	Moderate disorganization and displacement of cell bodies in the INL	Moderate disorganization of photoreceptor cell bodies in the ONL with displacement into the IS/OS region
	3	Severe disorganization of the INL	Severe disorganization of the ONL
Pyknosis	0	No pyknotic cells in the INL or GCL	No pyknotic cells in the ONL
	1	Singular pyknotic cell bodies (<10 in total) in the INL, singular pyknotic cell bodies in the GCL	Singular pyknotic cell bodies (<15 in total) in the ONL
	2	Scattered pyknotic cell bodies in the entire INL (>10 in total), the majority of cells non-pyknotic; singular pyknotic cell bodies in the GCL	Scattered pyknotic cell bodies in the entire ONL (>15 in total), the majority of cells non-pyknotic
	3	The majority of cell bodies in the INL and GCL pyknotic	The majority of cell bodies in the ONL pyknotic
Vacuolization	0	Normal vacuoles found in the outer part of the INL, and in the GCL; no vacuoles in the IPL	No vacuoles in the ONL
	1	Vacuoles found within the INL; no increase in size of normal vacuoles	Singular vacuoles found within the ONL
	2	Moderately increased number of vacuoles within the INL and GCL; vacuoles found in the IPL; no increased size of the normal vacuoles	Moderately increased number of vacuoles found within the ONL
	3	Severe increase in number of vacuoles within the INL, IPL, and GCL; increased size of the normal vacuoles	Severe increase in number of vacuoles found within the ONL disrupting the normal cellular architecture
OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.			

Quantification and Statistical Analysis

All morphological data from retinas examined 90 and 240 min after enucleation were compared with *in vivo* controls and analyzed using one-way ANOVA. In the culture experiment, morphological and LDH data from the 2 groups (cultured 48 h, 90 or 240 min after enucleation) was analyzed using Student's *t*-test. GraphPad Prism version 8.4.2 for Mac (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses, and $p < 0.05$ was considered significant.

For morphological analysis (H&E, TUNEL, and RBPMS), 4 tissue sections from each retinal specimen were photographed in a masked manner under the microscope using the $\times 20$ objective (Olympus BX53; Olympus, Münster, Germany). For each section, 3 photographs (1920×1200 pixels) were taken using a digital camera system (Olympus DP74), one from the very center of the section and one on each side, leaving approximately one image frame of space between photographs. Thus, for each staining/labeling, each retinal specimen generated 12 images, and each group 144 images to be analyzed. Furthermore, 24 images per staining/labeling derived from 2 normal porcine eyes were used as *in vivo* controls.

For quantification of overall retinal tissue damage (H&E staining), images were graded according to a tissue damage score (Ta-

ble 1): 0 representing normal morphology, 1 mild, 2 moderate, and 3 severe damage. The following parameters were evaluated: pyknosis, lamination, and vacuolization with the inner and outer retina graded separately. The average score for the inner and outer retina was calculated from the 3 parameters above and analyzed statistically. The tissue damage score was graded individually in a masked manner by 2 observers.

Since examination of the ganglion cell layer is precarious in H&E staining, the specific ganglion cell marker RBPMS mentioned above was used to ascertain the number of remaining ganglion cells. Labeled cells on each image were counted in a masked manner. Only completely labeled cells, positioned in the ganglion cell layer were counted. Debris and fragmented cellular structures were excluded.

TUNEL labeling was quantified in a masked manner using ImageJ (US National Institutes of Health, Bethesda, MD, USA; <https://imagej.nih.gov/ij/>). On each image, the inner (from the inner nuclear layer to the inner limiting membrane) and outer retina (from the outer plexiform layer to the outer segments) were delineated using the polygon selection tool, and TUNEL-positive cells automatically counted using the ImageJ-plugin, Macro cell counter [Maidana et al., 2015].

Results

Morphological Analysis of Tissue Damage after Enucleation

The in vivo controls, representing the normal retina, displayed the expected laminated appearance with no pyknotic cells in the cellular layers (Fig. 1A). Small vacuoles were present in the ganglion cell layer (GCL), as well as the in the outer part of the inner nuclear layer (INL). No vacuoles were detected in the outer nuclear layer (ONL).

Retinas examined 90 min after enucleation, in general, displayed well-defined lamination, but revealed tissue damage in the inner and outer retina in the form of loss of cell organization in the INL and ONL, an increase in vacuolization in the INL, and an increased amount of pyknotic cells when compared with the in vivo controls (Fig. 1B, D; tissue damage score inner retina 0.12 ± 0.25 mean $\pm$ SD, $p < 0.05$; outer retina 0.18 ± 0.22 , $p < 0.001$).

Retinas examined 240 min after enucleation displayed a highly significant increase in tissue damage compared with 90-min counterparts in the form of increased vacuolization, especially in the GCL and INL, increased pyknosis, and loss of cellular organization (Fig. 1C, D; inner retina 0.67 ± 0.32 , $p < 0.0001$; outer retina 0.34 ± 0.23 , $p < 0.0001$).

TUNEL staining of in vivo retinas displayed background fluorescence only with no cellular labeling (Fig. 1E). Occasional TUNEL-labeled cells could be seen in retinas examined 90 and 240 min after enucleation, however, only background fluorescence was present in the majority of images (Fig. 1F, G). Statistical analysis of the number of TUNEL-labeled cells revealed no significant difference when comparing the in vivo retinas and retinas examined 90 min after enucleation (Fig. 1H; inner retina 0.35 ± 0.67 ; outer retina 0.67 ± 3.41). Retinas examined 240 min after enucleation displayed a significant increase in labeled cells in the inner retina compared with the in vivo, as well as 90-min counterparts (Fig. 1H; inner retina 0.68 ± 1.52 , $p < 0.01$ and $p < 0.05$). No significant difference in labeled cells was seen in the outer retina (0.23 ± 0.51).

RBPMS immunohistochemistry revealed well-labeled, large cell bodies in the GCL in the in vivo retinas (Fig. 1I). A highly significant decline in labeled retinal ganglion cells was detected in retinas examined 90 and 240 min after enucleation compared to in vivo controls (Fig. 1I–L; in vivo 35.42 ± 16.70 ; 90 min 17.86 ± 12.09 ; 240 min 11.90 ± 7.31 , $p < 0.0001$). In addition, the number of labeled cells was significantly lower in retinas examined 240 min

after enucleation compared with 90-min counterparts ($p < 0.0001$).

Morphological and LDH Analysis of Tissue Damage after Enucleation and Tissue Culture

Retinal tissue explanted after 90 or 240 min and then cultured for 48 h, displayed a varying degree of disorganized lamination, pyknotic cells, as well as vacuoles in all 3 cellular layers in H&E-stained sections (Fig. 2A, B). A statistical analysis of the tissue damage score revealed no difference between the 2 groups (Fig. 2C; 90 min + culture: inner retina 0.77 ± 0.42 , outer retina 0.96 ± 0.62 ; 240 min + culture: inner retina 0.80 ± 0.31 , outer retina 0.91 ± 0.45).

TUNEL staining indicated apoptotic cells in all 3 nuclear layers in both culture groups, especially in the inner retina (Fig. 2D, E). Retinas cultured 240 min after enucleation displayed a significant increase of TUNEL-positive cells in the inner and outer retina compared with 90-min counterparts (Fig. 2F; 90 min + culture: inner retina 86.83 ± 67.74 , outer retina 56.99 ± 47.06 ; 240 min + culture: inner retina 171.78 ± 60.86 , outer retina 67.47 ± 38.54 , $p < 0.0001$ (inner), $p < 0.05$ (outer)).

A disparity between the 2 time points was also evident in RBPMS-labeled sections where explants cultured 240 min after enucleation displayed a strong significant decrease of ganglion cells when compared with 90-min counterparts (Fig. 2G–I; 90 min + culture: 9.51 ± 4.73 ; 240 min + culture: 4.62 ± 5.12 , $p < 0.0001$).

The level of the intracellular enzyme LDH was measured at 2, 24, and 48 h in the culture medium to assess cellular viability in the explants. LDH analysis revealed significantly higher activity (absorbance level) in culture medium derived from explants cultured 240 min after enucleation compared with 90-min counterparts at all time points (Fig. 3; 90 min + culture 2 h: 0.33 ± 0.12 ; 240 min + culture 2 h: 0.86 ± 0.52 , $p < 0.001$; 90 min + culture 24 h: 1.12 ± 0.49 ; 240 min + culture 24 h: 2.04 ± 0.52 , $p < 0.0001$; 90 min + culture 48 h: 2.20 ± 0.86 ; 240 min + culture 48 h: 2.53 ± 0.76 , $p < 0.05$).

Discussion

Retinal Tissue Damage after Enucleation Is Highly Time-Dependent

In this paper, we have explored the impact of time after enucleation in relation to tissue damage in the adult porcine retina. We focused our exploration on overall retinal morphological damage and apoptosis in the inner and

Fig. 1. Adult porcine retinal explants examined 90 or 240 min after enucleation. **A–C** Overall morphology; H&E staining. **A** Adult in vivo control, showing normal lamination of the retinal layers. **B** Retinal explant examined 90 min after enucleation, revealing signs of minor tissue damage in the inner and outer retina. **C** Retina examined 240 min after enucleation, with increased vacuolization, loss of cell organization and pyknotic cells compared to the 90-min counterparts. **D** Statistical analysis (one-way ANOVA) of the tissue damage score in the inner and outer retina. **E–G** Apoptosis; TUNEL staining. **E** Adult in vivo control, with no apoptotic cells. **F, G** Retinal explants examined 90 and 240 min after enucleation, revealing 1 apoptotic cell each (arrows). **H** Statistical analysis (one-way ANOVA) of the number of labeled cells in the inner and out-

er retina. **I–K** Ganglion cells; RBPMS immunohistochemistry. **I** Adult in vivo control, showing well labeled ganglion cells in the ganglion cell layer. **J, K** Retinal explants examined 90 and 240 min after enucleation, revealing a decline of labeled ganglion cells compared to the in vivo control. The retina examined 240 min after enucleation, shows a decrease of labeled ganglion cells compared to the 90 min-counterparts. **L** Statistical analysis (one-way ANOVA) of the number of labeled cells. **D, H, L** Bars represent mean values, error bars SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer. Scale bars, 100 μ m.

Fig. 2. Adult porcine retinal explants cultured for 48 h 90 or 240 min after enucleation. **A, B** Overall morphology; H&E staining. Both retinal explants show signs of a disorganization of cell bodies, scattered pyknotic cell bodies, and vacuoles. **C** Statistical analysis (Student's *t*-test) of the tissue damage score in the inner and outer retina. **D, E** Apoptosis; TUNEL staining. Retinal explants show apoptotic cells in all 3 nuclear layers. **F** Statistical analysis (Student's *t*-test) of the number of labeled cells in the inner and outer retina. **G, H** Ganglion cells; RBPMS immunohistochemistry. Reti-

nal explant cultured 240 min after enucleation for 48 h displays loss of ganglion cells compared with 90-min counterpart. **I** Statistical analysis (Student's *t*-test) of the number of labeled cells. **C, F, I** Bars represent mean values, error bars SD. * $p < 0.05$; **** $p < 0.0001$. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer. Scale bars, 100 μ m.

Fig. 3. Statistical analysis (Student's *t*-test) of the cell damage marker lactate dehydrogenase (LDH) in culture medium derived from retinal explants cultured 90 or 240 min for 2, 12, and 24 h. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$. Bars represent mean values, error bars SD. Time-dependent retinal tissue damage after enucleation.

outer retina and used immunohistochemistry to explore ganglion cell loss specifically. The results show that with the exception of ganglion cells, the retina is relatively well preserved 90 min after enucleation, whereas profound changes in the inner and outer retina are seen after 240 min. Enucleation of the eye induces global ischemia, and the increase in tissue damage seen 240 min after enucleation corresponds well to that reported in previous *in vivo* experiments where 90 min of ischemia have been reported to be well tolerated whereas 240 min are detrimental [Hayreh et al., 1980, 2004].

Even though retinas examined 90 min after enucleation displayed only minimal morphological deterioration and apoptosis, RBPMS labeling of ganglion cells was significantly decreased when compared to *in vivo* controls. In addition to global ischemia, the enucleation paradigm also includes transection of the optic nerve, which is well-known to produce ganglion cell death. However, this phenomenon is usually not detectable until after longer time periods [Watanabe and Fukuda, 2002]. When isolating the tissue prior to fixation, additional axonal trauma is induced within the retina, more central to the ganglion cell body. Therefore, we cannot exclude the possibility that the loss of RBPMS labeling in the ganglion cells 90 min after enucleation is primarily the result of axonal trauma and not ischemia.

Our results are at least in part in contrast to earlier findings concerning time-dependency after enucleation and may, therefore, be relevant to experimental research involving the large animal adult retina. Reinhard et al.

[2016] reported that ganglion cell activity in the mini-pig retina was maintained for at least 50 h after enucleation when the tissue was kept under hypothermic conditions at 4°C. Similarly, Schultheiss et al. [2016] found that hypothermia prolonged ganglion cell function and preserved the retinal structure for up to 340 min in bovine eyes after enucleation. To minimize tissue damage, we ensured that our eyes were kept cold at 4°C after enucleation until the time of dissection. In accordance with the previous studies, our retinas examined 240 min after enucleation displayed only a minimal increase in TUNEL detectable apoptosis compared with 90-minute counterparts, however, overall morphology and ganglion cell survival were profoundly affected, as evident in H&E-stained sections and RBPMS immunohistochemistry, suggesting that at least in our experimental setup, hypothermia failed to protect the retina 240 min after enucleation.

Time after Enucleation Has a Significant Impact on Retinal Tissue Damage in vitro

To further elucidate any possible extended time-dependent effects *in vitro*, we examined retinas harvested 90 or 240 min after enucleation with subsequent culturing for 48 h. The *in vitro* retinal explant setup is increasingly used for a wide array of research activities, including disease mechanism exploration, pharmacological intervention as well as transplantation [Engelsberg and Ghosh, 2007; Rettinger and Wang, 2018]. Immature retinal porcine tissue can be cultured for extended time periods and even show much of the normal development, however, the adult retina degenerates rapidly within the *in vitro* environment, which has been attributed to axotomy and the loss of retinal pigment epithelium-photoreceptor contact [Winkler et al., 2002; Engelsberg et al., 2005]. We have previously shown that cell survival in adult retinal porcine explants, including ganglion cells and photoreceptors, can be significantly enhanced by placing the explant with the inner limiting membrane facing the culture membrane [Taylor et al., 2014]. To limit the culture-induced effect, we thus used this method for the culture experiment.

The cultured retinas of both groups displayed a similar degree of overall tissue damage in H&E staining. However, a strong significant increase in TUNEL-labeled cells in the inner retina combined with profound loss of ganglion cells in the 240-min group indicates that the initial discrepancy in tissue damage discussed above is indeed extended *in vitro*. The increased tissue damage state of retinas 240 min after enucleation was further confirmed in the culture medium analysis in which the 240-min ex-

plants already after 2 h of culture displayed a highly significant increase in LDH activity. The implication of these findings is relevant to a range of research involving large animal adult retinal tissue experiments and may be of particular interest for explorations in vitro.

To summarize, we here show that time after enucleation is a crucial factor for retinal tissue survival in a large animal model. The adult porcine retina displays profound tissue damage 240 min after enucleation, whereas retinas examined after 90 min remain relatively intact, and this discrepancy is extended when the tissue is placed under culture conditions. These time points are in accordance with previous in vivo experiments and should be considered in experiments involving the ex vivo adult porcine retina.

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References

- Åkerström B, Cederlund M, Bergwik J, Manouchehrian O, Arnér K, Taylor IH, et al. The Role of Mitochondria, Oxidative Stress, and the Radical-binding Protein A1M in Cultured Porcine Retina. *Curr Eye Res*. 2017 Jun;42(6): 948–61.
- Engelsberg K, Ghosh F. Transplantation of cultured adult porcine full-thickness retina. *Cell Transplant*. 2007 Jan;16(1):31–9.
- Engelsberg K, Johansson K, Ghosh F. Development of the embryonic porcine neuroretina in vitro. *Ophthalmic Res*. 2005 Mar–Apr;37(2): 104–11.
- Hayreh SS, Kolder HE, Weingeist TA. Central retinal artery occlusion and retinal tolerance time. *Ophthalmology*. 1980 Jan;87(1):75–8.
- Hayreh SS, Zimmerman MB, Kimura A, Sanon A. Central retinal artery occlusion. Retinal survival time. *Exp Eye Res*. 2004 Mar;78(3):723–36.
- Maidana DE, Tsoka P, Tian B, Dib B, Matsumoto H, Kataoka K, et al. A novel ImageJ macro for automated cell death quantitation in the retina. *Invest Ophthalmol Vis Sci*. 2015 Oct; 56(11):6701–8.
- Reinhard K, Mutter M, Gustafsson E, Gustafsson L, Vaegler M, Schultheiss M, et al. Hypothermia promotes survival of ischemic retinal ganglion cells. *Invest Ophthalmol Vis Sci*. 2016 Feb;57(2):658–63.
- Rettinger CL, Wang HC. Current Advancements in the development and characterization of full-thickness adult neuroretina organotypic culture systems. *Cells Tissues Organs*. 2018; 206(3):119–32.
- Rodriguez AR, de Sevilla Müller LP, Brecha NC. The RNA binding protein RBPMs is a selective marker of ganglion cells in the mammalian retina. *J Comp Neurol*. 2014 Apr;522(6): 1411–43.
- Schnichels S, Kiebler T, Hurst J, Maliha AM, Löscher M, Dick HB, et al. Retinal Organ Cultures as Alternative Research Models. *Altern Lab Anim*. 2019 Mar;47(1):19–29.
- Schultheiss M, Schnichels S, Hermann T, Hurst J, Feldkaemper M, Arango-Gonzalez B, et al. Hypothermia Protects and Prolongs the Tolerance Time of Retinal Ganglion Cells against Ischemia. *PLoS One*. 2016 Feb;11(2): e0148616.
- Taylor L, Arnér K, Engelsberg K, Ghosh F. Effects of glial cell line-derived neurotrophic factor on the cultured adult full-thickness porcine retina. *Curr Eye Res*. 2013 Apr;38(4):503–15.
- Taylor L, Arnér K, Taylor IH, Ghosh F. Feet on the ground: physical support of the inner retina is a strong determinant for cell survival and structural preservation in vitro. *Invest Ophthalmol Vis Sci*. 2014 Apr;55(4):2200–13.
- Watanabe M, Fukuda Y. Survival and axonal regeneration of retinal ganglion cells in adult cats. *Prog Retin Eye Res*. 2002 Nov;21(6):529–53.
- Winkler J, Hagelstein S, Rohde M, Laqua H. Cellular and cytoskeletal dynamics within organ cultures of porcine neuroretina. *Exp Eye Res*. 2002 Jun;74(6):777–88.

Statement of Ethics

All procedures and handling of animals were in accordance with the guidelines and requirements of the Government Committee on Animal Experimentation at Lund University and also complied with the ARVO guidelines for animal experimentation.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

F.S.: Conception, experimentation, data collection and analysis, manuscript preparation, correspondence. B.Å.: Conception, data analysis (LDH), proof reading. F.G.: Conception, data collection and analysis, manuscript preparation.