tvst

Retina

Long-Term Porcine Retina Explants as an Alternative to In Vivo Experimentation

Maria Weller[1], Brigitte Müller[1], and Knut Stieger[1]

[1] Department of Ophthalmology, Justus-Liebig-University Giessen, Germany

Correspondence: Maria Weller, Department of Ophthalmology, Justus-Liebig-University Giessen, Aulweg 123, Giessen 35392, Germany. e-mail: maria.weller@augen.med.uni-giessen.de

Received: May 26, 2023
Accepted: January 24, 2024
Published: March 13, 2024

Keywords: tissue culture; explant culture; porcine retina; photoreceptors; rod bipolar cells

Citation: Weller M, Müller B, Stieger K. Long-term porcine retina explants as an alternative to in vivo experimentation. Transl Vis Sci Technol. 2024;13(3):9, https://doi.org/10.1167/tvst.13.3.9

Purpose: The porcine retina represents an optimal model system to study treatment approaches for inherited retinal dystrophies owing to close anatomical similarities to the human retina, including a cone enriched visual streak. The aim of this work was to establish a protocol to keep explants in culture for up to 28 days with good morphological preservation.

Methods: Two to four retina explants per eye were obtained from the central part of the retina and transferred onto a membrane insert with the photoreceptors facing down. Different medium compositions using Neurobasal-A medium containing 100 or 450 mg/dL glucose and combinations of fetal calf serum, B-27 with or without insulin and N-2 were tested. We developed a tissue quality score with robust markers for different retinal cell types (protein kinase C alpha, peanut agglutinin and 4′,6-diamidino-2-phenylindol).

Results: Retinae were kept until 28 days with only little degradation. The best results were attained using Neurobasal-A medium containing 100 mg/dL glucose supplemented with B-27 containing insulin and N-2. For an easy preparation process, it is necessary to minimize transport time and keep the eyes on ice until dissected. Heat-mediated decontamination by the butcher has to be avoided.

Conclusions: Using a standardized protocol, porcine retina explants represent an easy to handle intermediate model between in vitro and in vivo experimentation. This model system is robustly reproducible and contributes to the implementation of the 3R principle to minimize animal experimentation.

Translational Relevance: This model can be used to test future therapeutic approaches for inherited retinal dystrophies.

Introduction

Inherited retinal diseases comprise a group of disorders together affecting approximately 1 in 3000 to 4000 people worldwide.[1] In addition, acquired retinal diseases such as age-related macular degeneration affect approximately one-third of all people older than 70 years. The human retina has unique features compared with other species, including the cone enriched central macular region facilitating higher visual functions, such as visual acuity and color vision. Consequently, diseases affecting the cones immediately affect the central visual senses of the patients and represent a high burden.

Research into the pathogenesis and development of treatment approaches for cone-mediated diseases is hampered owing to the lack of appropriate animal model systems. In rodents for example, the cones are dispersed evenly throughout the entire retina, whereas the retina is generally rod dominant.[2,3] However, especially mice are a very-well established model system and can be genetically modified easily, which led to the development of several models for cone dystrophies.[2,4] Other species that can be used to model cone dystrophies are zebrafish, dogs, sheep, nonhuman primates, and pigs.[3,5–9] The porcine retina represents a valuable model, because of the anatomical similarities with human eyes and especially the cone-rich central region called the visual streak,[10,11] which can be seen as a

model for the human macula. However, breeding and maintaining pigs is a complex and expensive task and can be accomplished only at a few sites around the world.

Therefore, an intermediary system such as the organotypic explant culture has been developed and used in a variety of different applications.[4,12–21] In most of these studies, organotypic explant cultures have been kept for 7 to 14 days in culture, because the morphological structures at later time points start to deteriorate massively.[22,23] Usually obtained from a butcher or slaughter, the eyeballs are dissected immediately and the retina taken into a culture dish using membrane-based culturing systems. Differences between studies include the orientation of the retina on the membrane[24,25] and several co-culturing options, for example, with the retinal pigment epithelium (RPE).[13,22,25]

Therapeutic approaches, especially for inherited retinal diseases, are often associated with gene transfer using viral delivery systems, most often using adeno-associated viruses (AAVs).[26,27] Using these vectors, the fastest onset of transgene expression in vivo has been reported to be approximately 2 weeks.[28,29] Therefore, to use the explant culture system as model for AAV transduction, retinae need to be kept for at least 15 to 20 days in good condition regarding the morphological preservation.

The aim of this study was to develop a robust and reproducible protocol allowing cultivation of the porcine retina in explant cultures for at least 15 to 20 days. We tested different media compositions and also paid particular attention to the early preparatory steps, that is, heat treatment by the butcher and transfer time. To compare the different conditions objectively, we developed a tissue quality score. We were able to optimize the protocol to cultivate explant retinae for up to 28 days with good morphological preservation.

Methods

Preparation of Porcine Organotypic Retina Explants

Porcine eyes were obtained from a local butcher immediately after sacrifice and were placed in a bottle of ice-cold transport buffer (40% DMEM [Anprotec, Bruckberg, Germany], 40% RPMI 1640 [Anprotec], 10% HEPES [250 mM; Carl Roth, Karlsruhe, Germany], and 10% Anti-anti [Thermo Fisher Scientific, Dreieich, Germany]) and kept on ice until all eyes were processed. Eyes were transported to the laboratory and processed as quickly as possible, but within 3 h after enucleation at the most. The dissection was performed under a laminar flow hood (Thermo Fisher Scientific, Schwerte, Germany). The intact eyes were freed of excessive tissue and washed in 70% ethanol (Carl Roth) and rinsed with double distilled H_2O afterward. Subsequently, cornea, lens, and vitreous were removed and the posterior eyecup was rinsed with phosphate-buffered saline (PBS; 0.1 M, sterile, self-prepared). To gain explants from the visual streak the periphery was removed, and the remaining strip was cut into three to four square pieces with a 3- to 4-mm edge length. Thereafter, the retina was carefully peeled from the underlying pigment epithelium while being placed in a large drop of medium. For this step, a raspatory (a spoon with a very thin edge) was placed at the edge of the tissue piece and carefully slid between retina and pigment epithelium, lifting up the retina. Finally, the explant was transferred from the raspatory to a drop of medium on the semipermeable membrane insert (polycarbonate, 0.4 µm pore size; SPL Life Sciences, Pocheon-si, Gyeonggi-do, South Korea) in a six-well format with the photoreceptors facing the membrane. Any excess of medium around the explant was carefully removed with a pipette. At least three explants per condition were taken from different eyes ($n = 3$). As controls, uncultured retinae were dissected the same way but fixed and frozen directly.

Culture of Porcine Organotypic Retina Explants

Explants were cultured with different media compositions (Table 1). Medium was exchanged completely every other day using 1200 µL/well. Explants were kept at 37°C and 5% CO_2 in an incubator.

Harvesting of the Tissue and Preparation of Cross-Sections

Every 4 days up to day 28, explants were harvested to check tissue integrity by immunohistochemistry. For this task, the insert was removed from the plate and transferred to a petri dish. The membrane was cut around the explant with a scalpel and transferred to a 6-well plate containing 4% paraformaldehyde (Merck, Darmstadt, Germany) in PBS while taking extra care that the explant remains on the membrane. After fixation for 45 minutes, three washing steps with 0.1 M PBS, and immersion with 10% and 20% sucrose (Serva, Heidelberg, Germany) in 0.1 M PBS for 2 h each and with 30% sucrose in PBS overnight followed. Explants were embedded in Tissue Tek (Leica Biosys-

Table 1. Components Used for the Different Culture Media

Name of the Medium	Glucose (mg/dL)	FCS	B-27 Containing Insulin	B-27 Without Insulin	N-2
		10%	2%	2%	1%
+−100	100	x		x	
++100	100	x	x		
+−450	450	x		x	
++450	450	x	x		
−−100	100			x	
−+100	100		x		
−−450	450			x	
−+450	450		x		
−+100 N-2	100		x		x

The glucose level was adjusted by either mixing two Neurobasal-A media (Thermo Fisher Scientific; no glucose [Art. Nr.: A2477501] + 450 mg/dL glucose [Art. Nr.: 10888022]) or using Neurobasal-A with 450 mg/dL glucose alone. All media additionally contained 1% L-glutamine and antibiotic-antimycotic (5% for the first week, 1% for the remaining culture time). For the nomenclature of the media four criteria are important. The first digit of the name depicts if the medium contains FCS, the second digit if it contains insulin (as an ingredient of B-27) and the number represents the glucose level in milligrams per deciliter. If the medium contains N-2 supplement, N-2 follows the name.

Table 2. Primary and Secondary Antibodies Used for Immunohistochemistry

Antibody	Species	Reactivity	Dilution	Company
Primary antibodies				
Lectin PNA Conjugate Alexa Fluor 488	–	–	1:300	Molecular Probes, Eugene, OR, USA
PKCα	Mouse	–	1:300	Sigma Aldrich, Saint Louis, MO, USA
GFAP	Rabbit	–	1:1000	Merck, Darmstadt, Germany
Rhodopsin	Mouse	–	1:500	Millipore, Temecula, CA, USA
Secondary antibodies				
Alexa Fluor 594	Donkey	Mouse	1:500	Thermo Fisher GmbH, Dreieich, Germany
Alexa Fluor 488	Donkey	Rabbit	1:500	Thermo Fisher GmbH, Dreieich, Germany
Alexa Fluor 647	Donkey	Mouse	1:500	Thermo Fisher GmbH, Dreieich, Germany

GFAP, glial fibrillary acidic protein.

tems, Nussloch, Germany), frozen in liquid nitrogen and stored at −20°C. A microtome (MEV; Slee Medical, Mainz, Germany) was used to cut 14 µm cross-sections, taking five sections per Superfrost Plus slide (Langenbrinck, Emmendingen, Germany), and stored at −20°C.

Immunohistochemistry on Retinal Cross-Sections

For immunohistochemistry, several primary antibodies were used to identify different cell types (Table 2). First, the slides were air dried for 30 minutes. Afterward they were washed in 0.1 M PBS three times and blocked with blocking solution (10% normal donkey serum [Sigma Aldrich, Steinheim, Germany], 1% bovine serum albumin [Serva], and 0.5% Triton X-100 [Carl Roth] in 0.1 M PBS) for 45 minutes. After a quick rinse with PBS, they were incubated with primary antibody solution at room temperature overnight. The next day, the slides were washed in PBS three times and then incubated with secondary antibodies and 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies, Carlsbad, CA) for 90 minutes in the dark at room temperature. Next, the slides were again rinsed with PBS and covered with Fluorescence Mounting Medium (Agilent Dako, Santa Clara, CA) and a coverslip. Slides incubated without primary antibody served as negative controls.

Tissue Quality Score and Layer Thickness Measurements

The developed tissue quality score (Table 3) was used to quantify tissue integrity and compare different medium compositions and culture durations on cross

Table 3. Tissue Quality Score for Morphological Assessment of Immunohistochemistry-stained Retina Explants

	0	1	2
Rod bipolar cells	None or barely visible	Axon fragmented	Axon long and continuous
ONL and INL nuclei	None or severely pyknotic	Not fully round, more than 20% are pyknotic	No pyknosis, round shape
Cone inner segments	None	In >50% of the picture	Densely packed
Cone outer segments	None	At >50% of IS	At (almost) every IS

sections stained with cell specific antibodies. For evaluation, images were taken with the confocal microscope Fluoview FV10i (Olympus, Hamburg, Germany) from two different sections per explant and the same single grayscale frame was scored and measured for each category. An entire cryosection of an explant (approximately 4 mm) was imaged as an overview with ×10 magnification and the images used for assessing the quality of the explants were taken with ×60 magnification, are approximately 180 µm wide, and span the whole thickness of the retina. The layer thickness measurements were done in three locations of one frame of a DAPI-stained section with Fiji.[30] If the retina was too large to fit in one picture, a merge of two overlapping parts was performed with the Fiji plugin "stitching" before the measurements.[31]

Statistical Analysis

Statistical analysis was carried out in SigmaPlot (Systat, Erkrath, Germany). The groups were compared by One Way ANOVA. For inter group comparison we calculated the mean and standard deviation. A P value of less than 0.05 was considered statistically significant and levels of significance were defined as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

Finding the Optimal Culture Medium

To be able to find the most suitable culture medium, different compositions were tested regarding their effect on retinal integrity over time. For the evaluation of integrity, rod bipolar cells, cone photoreceptors, and the nuclei in the inner nuclear layer (INL) and outer nuclear layer (ONL) were analyzed, as protein kinase C alpha, peanut agglutinin (PNA), and DAPI are robust markers in porcine retina. In addition, the thickness of the entire retina as well as of the ONL was measured in DAPI stained sections. For the comparison of the different media, explants were harvested after 12 days in culture. Quantification was carried out using the newly developed tissue quality score in Table 3. For uncultured retina (day 0), the highest tissue quality score was defined as 2.

To find the optimal culture medium for long-term experimentation, we tried nine different combinations of supplemented Neurobasal-A medium as displayed in Table 1. Glucose, FCS, B-27, and N-2 are the differing ingredients.

Looking at rod bipolar cells of 12-day-old explants (Figs. 1B–J), one can clearly see a decrease in tissue integrity compared with uncultured retina (Fig. 1A). The axons are fragmented after culture with most media, only ++100, −+450, and −+100 N-2 show a slightly better preservation in that regard. Perikarya in their healthy shape can only be clearly visualized after culture with −+100 N-2. Comparing the tissue quality score of rod bipolar cells, it is significantly diminished after culture compared with uncultured retina ($P < 0.001$) regardless of the medium. For the different media there are clear tendencies to what is helping to preserve the integrity of the tissue. Media containing FCS seem to have a negative effect on tissue integrity, as does a high glucose level of 450 mg/dL (Fig. 1K). The medium −+100 N-2 shows a significantly higher tissue quality score than all other media, except −+450.

For the nuclei, −+100 and −+100 N-2 show the highest tissue quality score (Fig. 1L). For the media +−100 and ++450, there are almost no intact nuclei, and ONL and INL are almost indistinguishable (Figs. 1B and 1C).

Regarding the cones, −+100 N-2 is the most applicable medium as well. Outer and inner segments as well as synaptic end feet of the cones stained with PNA are discernable after 12 days for every medium except +−100 and ++450 (Figs. 2B–J). The outer segments are preserved best with the media ++100, −−450, −+100 and −+100 N-2. Inner segments are preserved best with the media −+100 and −+100 N-2. The tissue quality score for the cone outer segments is the highest after 12 days with the medium −+100 and

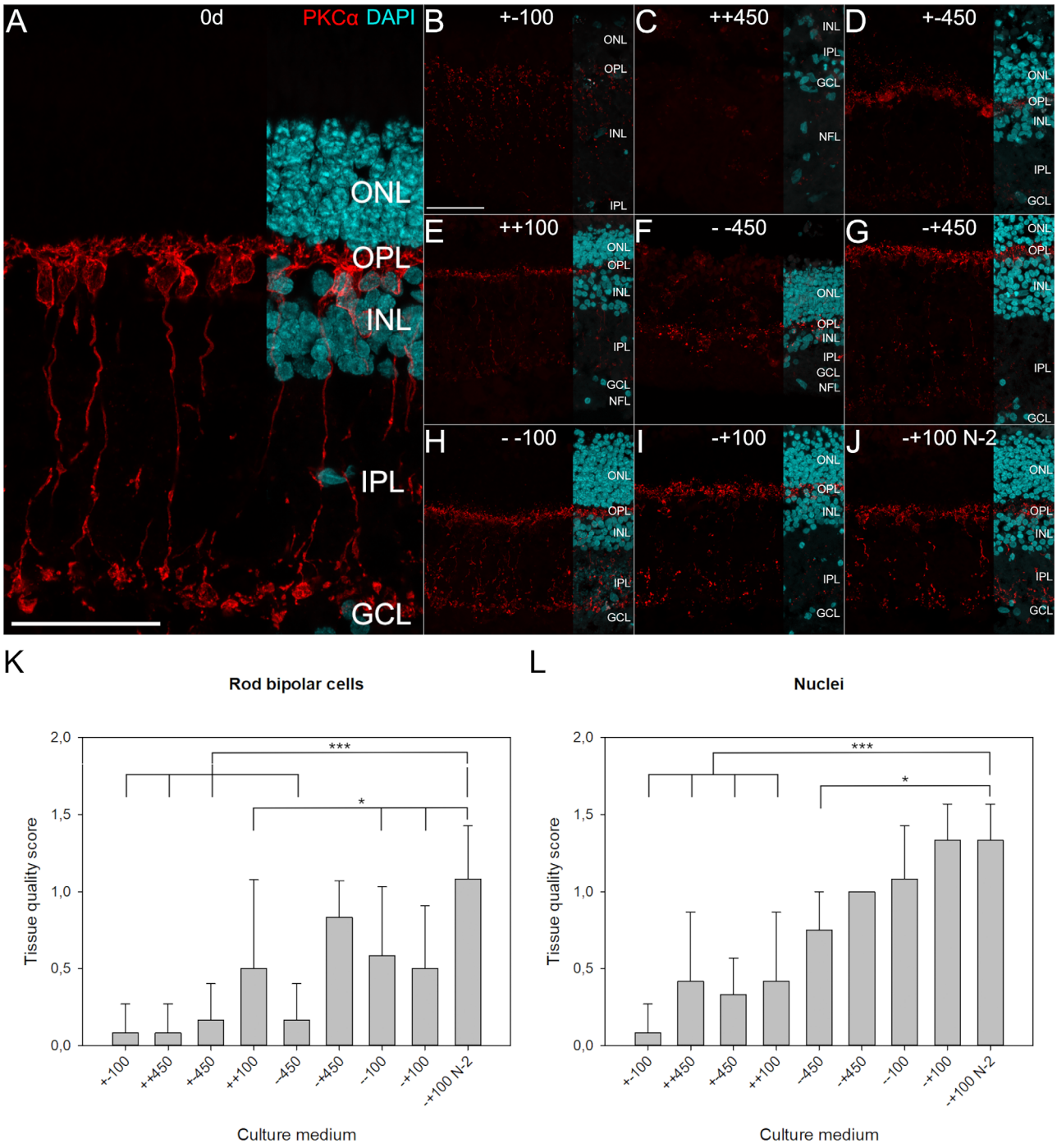

Figure 1. Evaluation of rod bipolar cells and nuclei in pig retina explant culture with different media for 12 days. (**A**–**J**) Rod bipolar cells were labeled with protein kinase C alpha (PKCα) (red) and nuclei with 4′,6-diamidino-2-phenylindole (DAPI) (blue). (**A**) Uncultured retina (d0). (**B**–**J**) Explants cultured for 12 days with different media. (**K**) Tissue quality score of rod bipolar cells after 12 days in culture with different media. Explants cultured with the medium −+100 N-2 showed a significantly higher tissue quality score than all other media except compared with −+ 450. (**L**) Tissue quality score of nuclei after 12 days in culture with different media. Explants cultured with the media −+100 or −+100 N-2 showed a significantly higher tissue quality score compared with explants cultured with +−100, ++450, +−450, ++100, and −−450. GCL, ganglion cell layer; IPL, inner plexiform layer; NFL, nerve fiber layer; OPL, outer plexiform layer. Scale bars, 50 µm. Values are mean ± standard deviation. $N = 3$; * $P < 0.05$ and *** $P < 0.001$.

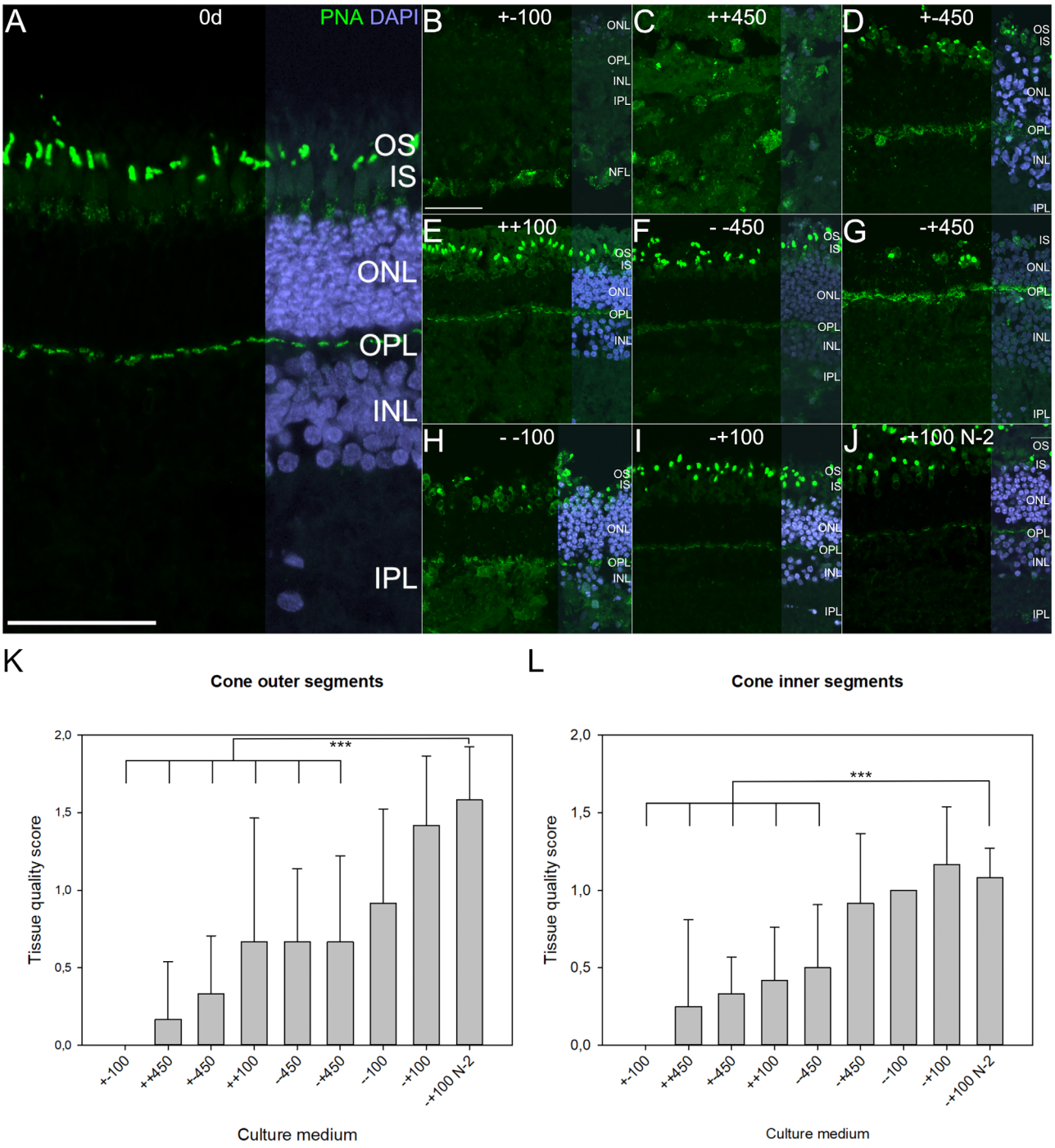

Figure 2. Evaluation of cone inner and outer segments after retina explant culture with different media for 12 days. (**A**–**J**) Cones were labeled with PNA (*green*) and nuclei with 4′,6-diamidino-2-phenylindole (DAPI) (purple). (**A**) Uncultured retina (d0). (**B**–**J**) Explants cultured for 12 days with different media. (**K**) Tissue quality score of cone outer segments after 12 days in culture with different media. Explants cultured with the medium −+100 N-2 showed a significantly higher tissue quality score than with all the other media except −−100 and −+100. (**L**) Tissue quality score of cone inner segments after 12 days in culture with different media. Explants cultured with the medium −+00 N-2 showed a significantly higher tissue quality score than explants cultured with +−100, ++450, +−450, ++100, and −−450. IS, inner segments; IPL, inner plexiform layer; NFL, nerve fiber layer; OPL, outer plexiform layer; OS, outer segments. Scale bars, 50 µm. Values are mean ± standard deviation. $N = 3$; *** $P < 0.001$.

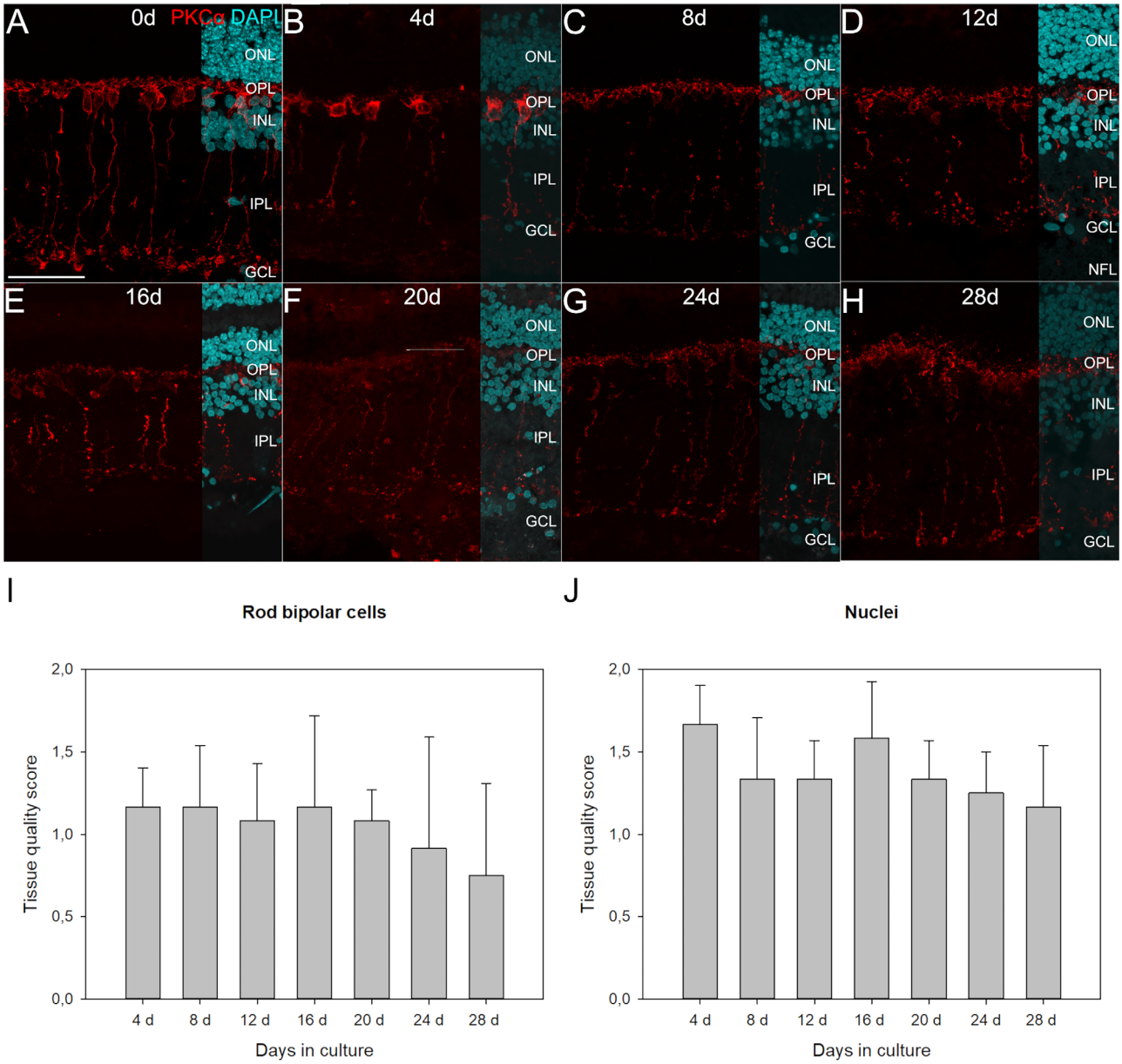

Figure 3. Evaluation of rod bipolar cells (RBs) and retinal nuclei after culture for 0 to 28 days with the medium −+100 N-2. (**A**–**H**) RBs were labeled with protein kinase C alpha (PKCα) (*red*) and nuclei with 4′,6-diamidino-2-phenylindole (DAPI) (*blue*). (**I**) Tissue quality score of RBs after culture for different length of time. There are no statistical differences between the time points displayed here. (**J**) Tissue quality score of retinal nuclei after culture for different length of time. There are no statistical differences between the time points displayed here. GCL, ganglion cell layer; IPL, inner plexiform layer; NFL, nerve fiber layer; OPL, outer plexiform layer. Scale bar, 50 µm. Values are mean ± standard deviation. $N = 3$.

the difference is highly significant compared with the other media except −−100 and −+100 N-2 (Fig. 2K). For the cone inner segments, the highest tissue quality score is achieved with −+100. Explants cultured with −+100 N-2 also show good preservation and the tissue quality score is significantly higher than with all other media, except −+450, −−100, and −+100 (Fig. 2L).

The thickness measurements of the ONL and the whole retina showed no clear change in thickness between different media. In addition, the changes that can be seen do not correspond to the results of the immunohistochemical analysis. After culture with all media but +−450 and −+100 N-2, the retina is significantly thinner than freshly explanted retina. The ONL is significantly thicker after culture with −+100

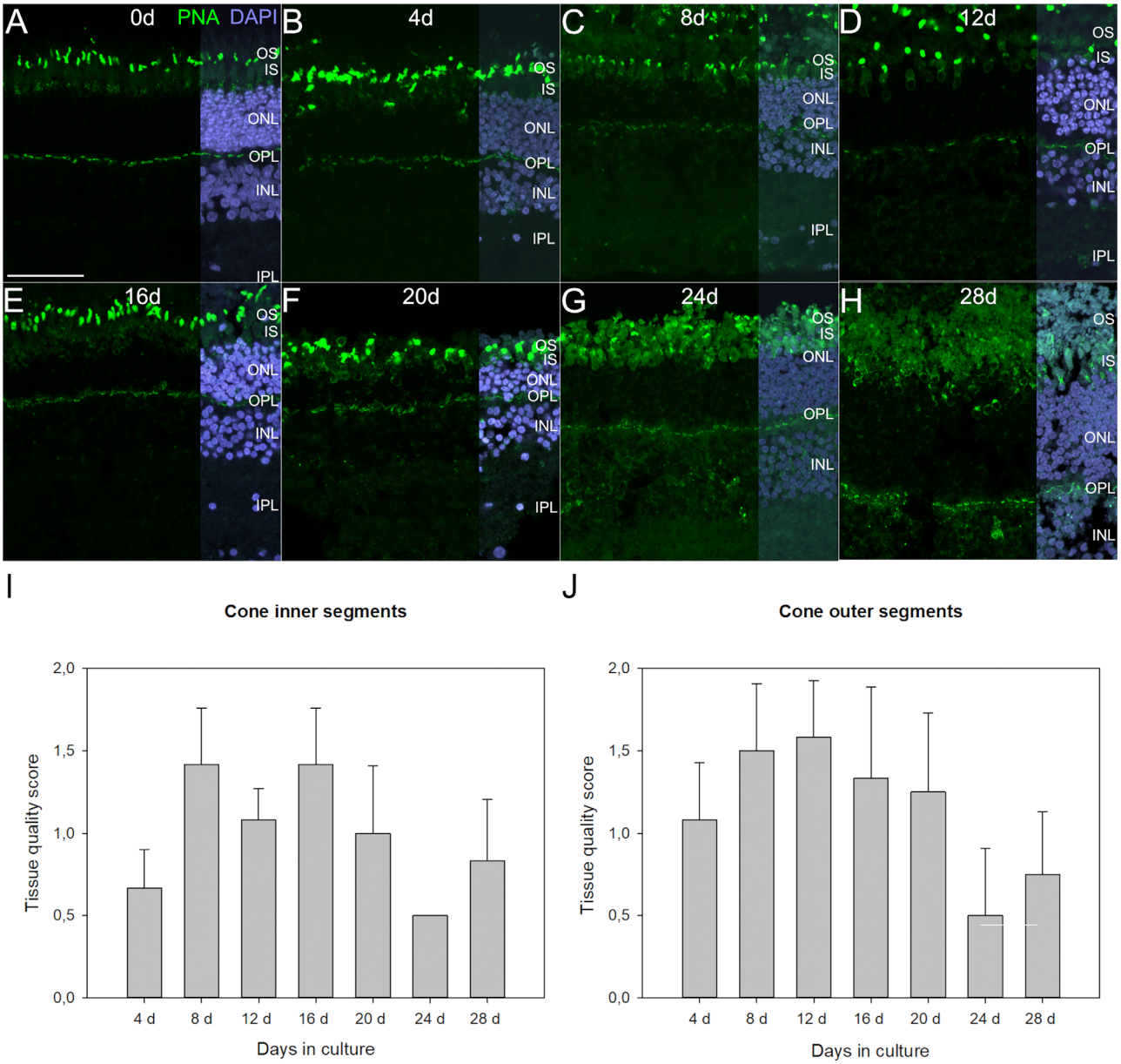

Figure 4. Evaluation of cone outer and inner segments after explant culture for 0 to 28 days with the medium −+100 N-2. (**A**–**H**) Cones were labeled with PNA (*green*) and nuclei with 4′,6-diamidino-2-phenylindole (DAPI) (*purple*). (**I**) Tissue quality score cone OS after culture for different length of time. (**J**) Tissue quality score of cone IS after culture for different length of time. IPL, inner plexiform layer; IS, inner segments; OPL, outer plexiform layer; OS, outer segments. Scale bar, 50 µm. Values are mean ± standard deviation. $N = 3$.

N-2 compared with all other media, except −−450, −−100, and −+100 (Supplementary Fig. S1).

To choose the optimal medium to continue the study with longer culture periods, we ranked the media according to the respective tissue quality score they reached in each category, added all the ranks and looked for the overall best scoring medium. The exact scores and measurements are shown in Supplementary Tables S1, S2, and S3. In brief, −+100 N-2 was ranked in the top three of each category and in sum showed the best tissue integrity after 12 days in culture.

Long-Term Experiments

After identifying −+100 N-2 as the best culture medium for tissue preservation, we studied tissue integrity in long-term cultures with this medium. Every 4 days up to day 28, explants were harvested and eventually analyzed with the help of the tissue quality score and layer thickness measurements.

Rod bipolar cells stained with antibodies against protein kinase C alpha showed good preservation after long-term culture with the medium −+100 N-2

(Figs. 3B–H). The axons look continuous and intact at all time points, only at day 8 they appear to be shorter and less strongly labelled between varicosities. Perikarya are clearly visible up to day 16 and the density of the cells stays almost the same over time. Nuclei are intact and appear healthy at every time point, and especially the INL loosens up a bit over time (Figs. 3B–H). The tissue quality score for the rod bipolar cells decreases a bit over time, but the differences between time points are not significant (Fig. 3I). Comparing to uncultured retina, the tissue quality score is significantly lower ($P < 0.05$). For the nuclei, the tissue quality score also decreases over time, but is similar to the results of rod bipolar cell staining, the differences between time points are not significant (Fig. 3J).

Cone photoreceptors are very well-preserved up to 20 days in culture with the medium −+100 N-2 (Figs. 4B–F). After 24 and 28 days in culture, cone inner segments are less neatly arranged and PNA gets delocalized into the cone perikarya at day 24 (Fig. 4H). Still, they are strongly labelled by PNA and the cone pedicles are readily recognizable (Figs. 4G and 4H). Although the tissue quality score for inner as well as for outer segments is not linearly declining with time, there are quite high interindividual differences at each time point, which resulted in high standard deviations (Figs. 4I and 4J). Generally, cone inner and outer segments are not well-maintained after 24 and 28 days in culture and significantly less preserved than in uncultured retina ($P < 0.001$).

The thickness of the retina in culture with −+100 N-2 varies over time. Beginning with 8 days in culture, it is significantly thinner than uncultured retina ($P < 0.05$). The ONL thickness, however, is not decreasing; at some time points, it is even higher than in fresh tissue (Supplementary Fig. S2). Additionally, we performed immunohistochemical analysis with antibodies against glial fibrillary acidic protein and rhodopsin to look for reactive gliosis and rod photoreceptor integrity. Activated Müller glia cells can be found in freshly explanted retina and very sparsely also after 4 day in culture with the medium −+100 N-2. There are no signs of reactive gliosis after longer periods of culture (Supplementary Fig. S3). A normal distribution of rhodopsin can be seen at all time points, but at day 28 after culture with −+100 N-2 (Supplementary Fig. S4).

Discussion

Porcine organotypic retina explant cultures provide great insight into disease mechanisms of inherited retinal diseases and can also be of use for studying future therapeutic approaches. To be able to use these cultures to their full potential, long term data of more than three weeks in culture are needed. In most previous studies, culture periods of 7 to 14 days were used.[22,23] After a comparison of different media on day 12 in culture, the most promising one was identified and longer culture periods were tested. We used a self-developed tissue quality score after immunohistochemistry to receive quantifiable data.

To start optimization of the protocol, an important step to consider is to keep the transport time to a minimum, because additional time without nutrients is detrimental to the retina.[18] Information regarding this early and crucial period is rarely found in the literature, but the general agreement is to keep the time as short as possible.[15,18,32] Therefore, we transport the eyes in a specialized transport buffer on ice. For an easy dissection process, especially for removing the vitreous body without damaging the retina, we found these cold conditions to be vital. To avoid additional stress and damage to the retina, we found that the butcher should not treat the eyes or the whole pig head with steam or directed combustion, because this process can denature proteins and alter the morphology of the retina.

When preparing the retina for explant culture, taking it together with the underlying RPE or without has been long discussed in the field, and different protocols have been published in the past.[13,22,25] Because the RPE and photoreceptors form a functional unit in vivo, it is a valid hypothesis that co-culturing would be beneficial. However, we did not observe a beneficial influence of the RPE when culturing the retina on top of an RPE. To mimic the function of the RPE, we use a semipermeable membrane, a procedure that is generally accepted in the field; almost all other groups use such membrane inserts.[12,15,22,24,25] We found that cutting the retina in small pieces and removing the retina from the underlying RPE/choroid using a raspatory worked optimally. Initially, we have tested a number of different methods, including a trepan stamp and the tweezers method.[25] We observed the best outcome when using a scalpel and this very thin raspatory to avoid tissue injury at the maximum level.

Different semipermeable membranes have been used in the literature to culture retinal explants.[12,15,33] In particular, membranes made of polycarbonate have a good outcome and allow culture periods up until 14 days without too much destruction. Here, we used semipermeable polycarbonate membrane inserts from SPL Life Sciences, which have not been used in pig explant culturing so far. We had obtained good experience with this type of membrane in mouse explant culturing[19,34] and, therefore, continued to use it herein.

Most groups use Neurobasal-A medium with several supplements to cultivate retina explants.[25,35,36] Keeping this in mind we explored different combinations of supplements and also varied the glucose concentration of the media, because too much glucose seems to be neurotoxic.[37,38] Variable ingredients were fetal calf serum (FCS), insulin as part of different types of commercially available B-27 supplement, N-2, and, as previously mentioned, glucose. Additionally, all tested media contained L-glutamine as essential amino acid and an antibiotic–antimycotic to prevent contaminants from growing. Results showed that FCS does not contribute to maintaining a good integrity of retina explants. Because FCS is not a chemically defined component, this result cannot be explained with certainty. However, the use of FCS in cell culture is controversial anyway. Serum-free media are to be preferred because every compound can be controlled and animal welfare is considered.[39–41] Furthermore, the B-27 supplement containing insulin showed better results in maintaining retinal tissue integrity. This result is consistent with other publications showing that insulin possesses antiapoptotic properties[42] and missing insulin plays a role in retinal ganglion cell degeneration.[43] N-2 as a supplement specifically designed for postmitotic neuronal cells also helps in preserving tissue integrity during retina explant culture. In total, explants cultured with the medium −+100 N-2 showed the best integrity after 12 days. Rod bipolar cells, cone inner and outer segments, and the nuclei in the ONL and INL looked well preserved. To the best of our knowledge, there is no other group that tested different glucose concentrations in the culture medium for retina explants.

To assess preservation of the retinal structures, we used standard rod, cone, bipolar, and Müller cell markers. To further describe the effect of ex vivo culturing, one could expand the analysis to ganglion cell markers such as RNA binding protein with multiple splicing or apoptosis assays such as terminal uridine nick-end labeling.[18,44,45] However, by using the presented marker and our tissue quality score, we were able to robustly and quantitatively assess structural preservation and to compare the different culturing conditions. A similar score was used by Svare et al.,[18] although other groups have described the preservation solely on a qualitative level, a method that is similarly suitable for developing protocols for explant cultures.

Measurements of the ONL thickness have shown that it only increases beginning at approximately day 24 and is mostly stable beforehand. The measurements of whole retina thickness were not that conclusive. The retinal thickness decreases with culture time, but this decrease is not linear. Some of the thinning can also have been caused by suboptimal explantation or individual physiological properties of the animal. Retinal remodeling can be an explanation for an increased ONL thickness as the nuclei become less densely packed and retinal layering can be disturbed by dislocated cells.[46–48] Also, the intermittent thickening of the retina is not corresponding with Müller cell swelling, because analysis of glial fibrillary acidic protein immunohistochemistry show no reactive gliosis after day 4 in culture.

To see if we can cultivate porcine retina explants for longer than 3 weeks, we used the same criteria as for identifying the best medium composition. The medium −+100 N-2 was chosen and we harvested explants every 4 days up to day 28 in vitro. The results showed that there is no significant difference between time points up to day 20; after that, the tissue quality decreases at a greater rate. However, the tissue quality score after 28 days in culture might still be acceptable for some experiments, depending on which cell type is being investigated. Nonetheless, it is better to choose the culture time appropriate to the experiment, keeping it as short as possible but as long as needed. Studies about AAV administration might require a culture time of approximately 3 weeks, whereas studies about age-related degeneration take longer than that. Another point to consider are therapeutics that have to be administered several times over the course of a few weeks,[49,50] which could now also be modelled by this porcine retina explant culture with longer culture periods.

When using an intermediary model such as the retina explant culture, which is made with post mortem tissue, one could also directly use human tissue. Several groups have published protocols and used human retina explants to address important questions regarding therapy development and degeneration processes.[4,23,36,50,51] However, there are several reasons that favor using the porcine retina as a model. First, pig eyes are readily available from slaughterhouses and can be obtained in large numbers. This is often not the case for human eyes, which renders this model more complex. Second, the pig model enters more and more into the spotlight in in vivo therapy development for inherited retinal diseases, including the retina explant culture from the same species in preliminary work, before entering in vivo experimentation increases robustness of the generated data and also favors the 3R argumentation to limit the number of animals in research.

In this study, we established a protocol for a porcine retina explant culture that can be used for long-term experiments, such as testing gene therapy approaches. Based on immunofluorescence data achieved on frozen

retinal cross sections we found that a culture time up to 28 days is possible with the proper medium composition. For most purposes (e.g., after AAV transduction), this morphological analysis should be sufficient to classify results, especially if fluorescently labelled expression constructs are used. If more detailed data are needed, gene expression or transcriptome studies could be conducted as well.[19,50] In addition, functional readout parameters could be generated with a multielectrode array, for example.[52,53] A porcine retina culture like this can be a valuable tool to test therapy strategies before moving to in vivo studies. Human retina explants would be even more beneficial to test such strategies, because there are no species differences to be accounted for, but the porcine model provides a more accessible and less expensive alternative, because the number of human eyes that can be used for research is very much limited. One limiting factor to be considered is, however, that one must test how this protocol translates to attempting explant cultures with the already degenerating retina of diseased animals. This determination would now be the next crucial step toward testing gene therapy approaches for inherited retinal dystrophies in an ex vivo model.

Acknowledgments

Disclosure: **M. Weller**, None; **B. Müller**, None; **K. Stieger**, None

References

1. Pulman J, Sahel J-A, Dalkara D. New editing tools for gene therapy in inherited retinal dystrophies. *CRISPR J*. 2022;5:377–388.
2. Schori C, Agbaga M-P, Brush RS, Ayyagari R, Grimm C, Samardzija M. Elovl4 5-bp deletion does not accelerate cone photoreceptor degeneration in an all-cone mouse. *PloS One*. 2018;13:e0190514.
3. Kostic C, Arsenijevic Y. Animal modelling for inherited central vision loss. *J Pathol*. 2016;238:300–310.
4. Eldred KC, Reh TA. Human retinal model systems: strengths, weaknesses, and future directions. *Dev Biol*. 2021;480:114–122.
5. Ail D, Nava D, Hwang IP, et al. Inducible nonhuman primate models of retinal degeneration for testing end-stage therapies. *Sci Adv*. 2023;9:eadg8163.
6. Link BA, Collery RF. Zebrafish models of retinal disease. *Annu Rev Vis Sci*. 2015;1:125–153.
7. Miyadera K. Mapping of canine models of inherited retinal diseases. *Adv Exp Med Biol*. 2018;1074:257–264.
8. Noel NCL, MacDonald IM, Allison WT. Zebrafish models of photoreceptor dysfunction and degeneration. *Biomolecules*. 2021;11:78.
9. Perkins BD. Zebrafish models of inherited retinal dystrophies. *J Transl Genet Genom*. 2022;6:95–110.
10. Mussolino C, Della Corte M, Rossi S, et al. AAV-mediated photoreceptor transduction of the pig cone-enriched retina. *Gene Ther*. 2011;18:637–645.
11. Slijkerman RWN, Song F, Astuti GDN, et al. The pros and cons of vertebrate animal models for functional and therapeutic research on inherited retinal dystrophies. *Prog Retin Eye Res*. 2015;48:137–159.
12. Schnichels S, Kiebler T, Hurst J, et al. Retinal organ cultures as alternative research models. *Altern Lab Anim ATLA*. 2019;47:19–29.
13. Di Lauro S, Rodriguez-Crespo D, Gayoso MJ, et al. A novel coculture model of porcine central neuroretina explants and retinal pigment epithelium cells. *Mol Vis*. 2016;22:243–253.
14. Maliha AM, Kuehn S, Hurst J, et al. Diminished apoptosis in hypoxic porcine retina explant cultures through hypothermia. *Sci Rep*. 2019;9:4898.
15. Rettinger CL, Wang H-C. Quantitative assessment of retina explant viability in a porcine ex vivo neuroretina model. *J Ocul Pharmacol Ther*. 2018;34:521–530.
16. Schnichels S, Blak M, Hurst J, et al. Establishment of a retinal hypoxia organ culture model. *Biol Open*. 2017;6:1056–1064.
17. Schnichels S, Schultheiss M, Klemm P, et al. Cyclosporine a protects retinal explants against hypoxia. *Int J Mol Sci*. 2021;22(19):10196.
18. Svare F, Åkerström B, Ghosh F. It's about time: time-dependent tissue damage in the adult porcine retina after enucleation. *Cells, Tissues, Organs*. 2021;210:58–65.
19. Müller B, Wagner F, Lorenz B, Stieger K. Organotypic cultures of adult mouse retina: morphologic changes and gene expression. *Invest Ophthalmol Vis Sci*. 2017;58:1930–1940.
20. Peynshaert K, Devoldere J, Forster V, et al. Toward smart design of retinal drug carriers: a novel bovine retinal explant model to study the barrier role of the vitreoretinal interface. *Drug Deliv*. 2017;24:1384–1394.
21. Pavlou M, Schön C, Occelli LM, et al. Novel AAV capsids for intravitreal gene therapy of photoreceptor disorders. *EMBO Mol Med*. 2021;13:e13392.
22. Schnichels S, Paquet-Durand F, Löscher M, et al. Retina in a dish: cell cultures, retinal explants and

animal models for common diseases of the retina. *Prog Retina Eye Res*. 2020:100880.
23. Rettinger CL, Wang H-C. Current advancements in the development and characterization of full-thickness adult neuroretina organotypic culture systems. *Cells, Tissues, Organs*. 2018;206:119–132.
24. 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;55:2200–2213.
25. Wagner N, Reinehr S, Gammel MR, et al. Novel porcine retina cultivation techniques provide improved photoreceptor preservation. *Front Neurol*. 2020;14:556700.
26. Allocca M, Doria M, Petrillo M, et al. Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J Clin Invest*. 2008;118:1955–1964.
27. McClements ME, MacLaren RE. Gene therapy for retinal disease. *Transl Res*. 2013;161:241–254.
28. McCarty DM. Self-complementary AAV vectors; advances and applications. *Mol Ther*. 2008;16:1648–1656.
29. Colella P, Auricchio A. Gene therapy of inherited retinopathies: a long and successful road from viral vectors to patients. *Hum Gene Ther*. 2012;23:796–807.
30. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat. Methods*. 2012;9:676–682.
31. Preibisch S, Saalfeld S, Tomancak P. Globally optimal stitching of tiled 3D microscopic image acquisitions. *Bioinformatics*. 2009;25:1463–1465.
32. Belhadj S, Tolone A, Christensen G, Das S, Chen Y, Paquet-Durand F. Long-term, serum-free cultivation of organotypic mouse retina explants with intact retinal pigment epithelium. *J. Vis. Exp.* 2020;165:e61868, doi:10.3791/61868.
33. 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;38:503–515.
34. Müller B, Serafin F, Laucke LL, Rheinhard W, Wimmer T, Stieger K. Characterization of double-strand break repair protein Ku80 location within the murine retina. *Invest Ophthalmol Vis Sci*. 2022;63:22.
35. Bell K, Wilding C, Funke S, et al. Neuroprotective effects of antibodies on retinal ganglion cells in an adolescent retina organ culture. *J Neurochem*. 2016;139:256–269.
36. Wiley LA, Burnight ER, Kaalberg EE, et al. Assessment of adeno-associated virus serotype tropism in human retinal explants. *Hum Gene Ther*. 2018;29:424–436.
37. Bahniwal M, Little JP, Klegeris A. High glucose enhances neurotoxicity and inflammatory cytokine secretion by stimulated human astrocytes. *Curr Alzheimer Res*. 2017;14:731–741.
38. Tomlinson DR, Gardiner NJ. Glucose neurotoxicity. *Nat Rev Neurosci*. 2008;9:36–45.
39. van der Valk J, Mellor D, Brands R, et al. The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicol In Vitro*. 2004;18:1–12.
40. van der Valk J, Brunner D, de Smet K, et al. Optimization of chemically defined cell culture media–replacing fetal bovine serum in mammalian in vitro methods. *Toxicol In Vitro*. 2010;24:1053–1063.
41. Hartung T, Balls M, Bardouille C, et al. Good cell culture practice. ECVAM good cell culture practice task force report 1. *Altern Lab Anim*. 2002;30:407–414.
42. Duenker N, Valenciano AI, Franke A, et al. Balance of pro-apoptotic transforming growth factor-beta and anti-apoptotic insulin effects in the control of cell death in the postnatal mouse retina. *Eur J Neurosci*. 2005;22:28–38.
43. Al Hussein Al Awamlh S, Wareham LK, Risner ML, Calkins DJ. Insulin signaling as a therapeutic target in glaucomatous neurodegeneration. *Int J Mol Sci*. 2021;22(9):4672.
44. Pereiro X, Ruzafa N, Urcola JH, Sharma SC, Vecino E. Differential distribution of RBPMS in pig, rat, and human retina after damage. *Int J Mol Sci*. 2020;21(23):9330.
45. Alarautalahti V, Ragauskas S, Hakkarainen JJ, et al. Viability of mouse retinal explant cultures assessed by preservation of functionality and morphology. *Invest Ophthalmol Vis Sci*. 2019;60:1914–1927.
46. Cuenca N, Fernández-Sánchez L, Campello L, et al. Cellular responses following retinal injuries and therapeutic approaches for neurodegenerative diseases. *Prog Retina Eye Res*. 2014;43:17–75.
47. Jones BW, Marc RE. Retinal remodeling during retinal degeneration. *Exp Eye Res*. 2005;81:123–137.
48. Marc RE, Jones BW. Retinal remodeling in inherited photoreceptor degenerations. *Mol Neurobiol*. 2003;28:139–148.
49. Sun Da, Sun W, Gao S-Q, et al. Effective gene therapy of Stargardt disease with PEG-ECO/pGRK1-ABCA4-S/MAR nanoparticles. *Mol Ther Nucleic Acids*. 2022;29:823–835.

50. Xi Z, Öztürk BE, Johnson ME, Turunç S, Stauffer WR, Byrne LC. Quantitative single-cell transcriptome-based ranking of engineered AAVs in human retinal explants. *Mol Ther Methods Clin Dev*. 2022;25:476–489.
51. Jones MA, Jadeja RN, Flandrin O, et al. Autonomous regulation of retinal insulin biosynthesis in diabetes. *Neuropeptides*. 2022;94:102258.
52. Field GD, Gauthier JL, Sher A, et al. Functional connectivity in the retina at the resolution of photoreceptors. *Nature*. 2010;467:673–677.
53. Fiscella M, Franke F, Farrow K, et al. Visual coding with a population of direction-selective neurons. *J Neurophysiol*. 2015;114:2485–2499.