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Retina

Beneficial and Detrimental Pressure-Related Effects on Inner Neurons in the Adult Porcine In Vitro Retina

Frida Svare[1] **and Fredrik Ghosh**[1]

[1] Department of Ophthalmology, Lund University, Lund, Sweden

Correspondence: Frida Svare, Department of Ophthalmology, Lund University, BMC D10, Klinikgatan 32, SE 22184 Lund, Sweden. e-mail: frida.svare_johanson@med.lu.se

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Purpose: To explore pressure-related effects in the adult porcine retina in vitro.

Methods: Retinal explants were subjected to 0, 10, 30, or 60 mmHg of pressure for 24 or 48 hours in culture. Overall tissue damage in sections was assessed by lactate dehydrogenase media levels, hematoxylin and eosin staining, and TUNEL staining. Inner retinal neurons were evaluated by protein kinase C alpha (rod bipolar cells), CHX10 (overall bipolar cell population), parvalbumin (amacrine cells), and RBPMS (ganglion cells) immunohistochemistry.

Results: All retinas kept in culture displayed increased pyknosis and apoptosis compared with directly fixed controls. The 10-mmHg explants displayed attenuation of overall tissue damage compared with the 0-, 30-, and 60-mmHg counterparts. No difference in the number of rod bipolar cells was seen in the 10-mmHg explants compared with directly fixed controls, whereas significantly fewer cells were detected in the remaining pressure groups. No difference in the number of ganglion cells in the 0-, 10-, and 60-mmHg groups was seen compared with directly fixed controls after 24 hours, whereas a lower number was found in the 30-mmHg counterpart. A decline of ganglion cells was found in the 0-, 10-, and 60-mmHg group after 48 hours, but no further decrease was seen in the 30-mmHg group. No differences were detected in overall bipolar and amacrine cells in the pressure groups after 24 hours compared with directly fixed controls.

Conclusions: A moderate amount of pressure attenuates culture-related retinal neurodegeneration. Rod bipolar cells are specifically vulnerable to excessive pressure.

Translational Relevance: These findings are relevant for glaucoma-related research.

Introduction

Various tissues within the body are constantly exposed to biomechanical forces such as pressure, stretch, torsion, twisting, and compression that provide cells with vital information about their surroundings to which they respond and adapt.[1] Biomechanical stimuli have gained increased interest in recent years due to elucidation of their importance in maintaining tissue homeostasis, as well as the important roles they play in pathological conditions.[2]

The interior of the normal eye, including the neuroretinal sheet, is constantly exposed to the intraocular pressure (IOP), which causes biomechanical stretch and compression of the tissue. The reason why the IOP exists has not yet been fully revealed; however, our understanding of the biomechanical system within the retina in which mechanosensitive cells rapidly respond to normal IOP fluctuations is progressively expanding.[1,3]

Elevated IOP is currently the leading risk factor for developing glaucoma, a neuropathy affecting the retinal ganglion cells (RGCs) and their axons, leading to characteristic structural damage in the optic nerve head and irreversible visual field loss.[4] Lowering the IOP by surgery or eye drops remains the only treatment for glaucoma and has been shown to at least partly slow its progression.[4,5] Currently, no definitive cure for the disease exists, and the exact cellular mechanisms behind the progression of the disease remain unknown.

Pressure-related effects on RGCs have been well explored in several in vivo studies indicating that the primary insult in glaucoma occurs in RGC axons, followed by degeneration and decline of RGC function and loss of ganglion cell bodies, as well as their axons.[6,7] Interestingly, a few studies have shown that other retinal neuronal subtypes located in the inner nuclear layer (INL), as well as glial cells, are affected early and have been implicated as sources of primary pressure-related insult.[8–13]

To further elucidate dynamic cellular changes in glaucoma, several in vitro studies, ranging from primary cell to eye cups, have been conducted.[14–17] Explanting retinal full-thickness pieces on a rigid culture membrane enables isolation of the pressure component on the tissue and precise control of pressure levels.[15] Most in vitro studies have explored pressure-related effects on the retinal tissue in small animal models, and the number of experiments involving larger animals is limited. In this study, we wanted to isolate and explore the effects of compressive force caused by pressure on a retina more resembling the human one. We also wanted to perform a comprehensive analysis of morphological changes in the three inner retinal neuronal subtypes involved in retinal signal transmission, with the goal of identifying detrimental pressure-related effects in bipolar cells, as well as in amacrine and ganglion cells. Finally, we wanted to investigate any possible beneficial effects related to pressure with the hypothesis that a moderate amount of pressure may support neuronal viability and be important for retinal homeostasis within the culture environment.

The porcine eye shares many similarities with the human counterpart, including size, an enriched cone area in the retina, and the presence of retinal vasculature.[18,19] To investigate the impact of pressure on the tissue, we used adult porcine retinal explants subjected to 0 mmHg (atmospheric pressure only); 10 mmHg, resembling the normal IOP; 30 mmHg, corresponding to conditions in primary open-angle glaucoma; and 60 mmHg, resembling acute glaucoma.[20] We report beneficial as well as detrimental effects.

Materials and Methods

Animals and Retinal Explant Culture

All procedures and animal handling were in accordance with the guidelines and requirements of the Government Committee on Animal Experimentation at Lund University (permit 8914-19) and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. After transportation from a local breeder to the Biomedical Centre at Lund University, young adult pigs (*Sus scrofa domesticus*) 4 to 6 months old were killed after sedation by an intravenous overdose of sodium pentobarbital (Apoteket, Umeå, Sweden). Both eyes were immediately enucleated, placed in a vial containing CO_2 Independent Medium (Invitrogen, Paisley, UK) at 4°C on ice, and moved to the laboratory where they were dissected as follows. The anterior segment was removed by sharp incision in the pars plana 360°, and the vitreous was 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 visual streak on the temporal and nasal side of the optic nerve head between the three major vascular arcades as described previously.[21]

Retinal pieces were explanted onto Millicell PCF Culture Plate Inserts (0.4-µm pore size, one retinal piece/well; MilliporeSigma, Billerica, MA), with the inner retina facing the membrane according to a previously published protocol.[22] This protocol was chosen to optimize tissue survival in vitro. Each explant was cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12; Invitrogen), including 3.151 mg/mL glucose, supplemented with 10% fetal bovine serum to support cellular viability (Sigma-Aldrich, St. Louis, MO) and 1% antibiotics (2 mM L-glutamine, 100 U/mL penicillin, and 100 ng/mL streptomycin (Sigm-Aldrich). Then, 1.5 mL medium was distributed into the culture wells 90 minutes prior to explantation of the retinal tissue to reach gas and temperature equilibrium.[23]

Pressure System

Retinal explants were cultured within an acrylic glass pressure chamber (AGP-1000, Strex Cell, San Diego, CA) which was placed inside an incubator at 37°C. Explants were cultured in two six-well plates inside the chamber on a raised platform with sterilized water underneath in order to maintain humidity. A control unit regulating the chamber pressure was placed in the room outside the incubator and was connected to the chamber within the incubator by plastic tubes. Gas into the control unit was obtained by an inflow tube, allowing air from the room to flow through the control unit and back into the pressure chamber placed within the incubator. The control unit was used to set the pressure and also to set air

exchange within the chamber every 30 minutes. The pressure within the chamber, which was monitored on the control unit, was found to be within ±1 mmHg of the set pressure, also during the 30 seconds of air exchange. This system allowed the retinal explants to be subjected to atmospheric pressure with the addition of pressure resulting from compression by the inflowing gas.

Groups

Four pressure groups were investigated and subjected to a pressure of 0 (atmospheric pressure only), 10, 30, or 60 mmHg for either 24 or 48 hours of tissue culture inside a pressurized chamber (see above). In each pressure group, explants from the left eye from each pig were subjected to pressure for 24 hours, and explants from the contralateral eye were subjected to pressure for 48 hours. In the 60-mmHg group, two explants were accidentally cultured with the outer retina facing the membrane and were therefore excluded. Ten to 12 explants were investigated for each pressure at each time point. At 24 hours, 12 explants were investigated in the 0-mmHg group; 12 explants in the 10-mmHg group; 12 explants in the 30-mmHg group; and 10 explants in the 60-mmHg group. At 48 hours, 12 explants were investigated in the 0-mmHg group; 12 explants in the 10-mmHg group; 12 explants in the 30-mmHg group; and 12 explants in the 60-mmHg group. This procedure generated 94 retinal explants in total. In addition, six retinal pieces from two different eyes were fixed immediately after enucleation and explantation and were used as controls without further culturing.

Histology

After 24 or 48 hours of culture, explants were fixed in 4% paraformaldehyde in 0.1-M phosphate buffer, pH 7.2, for 2 hours at room temperature. The directly fixed control retinas were processed in the same manner immediately after explantation. After fixation, the explants were infiltrated with 0.1-M Sörensen's medium with increasing concentrations of sucrose up to 25%. Cultured and directly fixed control retinal explants were then embedded in egg albumin/gelatin medium and cryosectioned at 12 µm, and the tissue sections were transferred to glass slides. For overall morphological assessment, sections on slides 1, 10, 20, and 25 were stained with hematoxylin and eosin (H&E) and slides 10 and 20 were used for analysis. For immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), two slides from the central part of each explant were used.

For immunohistochemistry, sections from cultured and control retinas were rinsed three times with phosphate-buffered saline (PBS) and then incubated with PBS buffer containing 0.25% Triton X-100 and 1% bovine serum albumin, for 45 minutes at room temperature. Together with the primary antibody, the sections were incubated overnight at 4°C and rinsed in PBS, followed by incubation for 45 minutes together with the secondary antibody Anti-Mouse IgG (Fab specific)-FITC antibody and/or Rhodamine Red-X-AffiniPure F(ab′)2 antibody (FITC antibody; Jackson ImmunoResearch Laboratories, West Grove, PA) and rhodamine antibody (Nordic BioSite AB, Täby, Sweden). Finally, slides containing the sections were mounted in VECTASHIELD Anti-Fade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Negative control experiments were performed as above but without the respective primary antibody. A rabbit polyclonal antibody raised against protein kinase C alpha (PKC-α; diluted 1:200; Sigma-Aldrich) was used that has been found to label rod bipolar cells in the porcine retina.[24] As no single antibody can be used to label cone bipolar cells specifically, we used a mouse monoclonal antibody raised against CHX10 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), which has been shown to label the entire bipolar population in mice (rod and cone bipolar cells).[25] A mouse monoclonal antibody raised against parvalbumin (1:1000; MilliporeSigma) was used to assess AII amacrine cells, which are primarily postsynaptic to rod bipolar cells.[26] In addition, a rabbit polyclonal antibody raised against RNA-binding protein with multiple splicing (RBPMS; 1:200; Abcam, Cambridge, UK) was used for the identification of RGCs.[27] To detect apoptotic cells, TUNEL staining was performed using the TMR Red In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany).

LDH Assay

For assessment of overall cell viability, media from the four pressure groups were sampled after 24 or 48 hours 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) according to the manufacturer's instructions. Three media samples per explant were used for analysis.

Morphological Analysis

For morphological analysis, two tissue sections from each retinal explant were photographed in a light microscope using the 20× objective (BX53; Olympus, Tokyo, Japan). For each section, three photographs 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 and labeling, each retinal explant generated six images, and each group generated 60 to 72 images to be analyzed. The directly fixed control explants generated 18 images from six different central sections per staining/labeling.

All images were analyzed in a blinded manner. For evaluation of overall tissue damage, the amount of pyknotic cells in the ganglion cell layer (GCL), INL, and outer nuclear layer (ONL) were graded in H&E-stained sections. The inner retina (GCL/INL) and outer retina (ONL) were graded separately. Images were graded on a scale from 0 to 3, where Grade 0 represented the normal retina with no pyknotic cells; Grade 1, fewer than 10 pyknotic cell bodies in the GCL/INL and fewer than 15 in the ONL; Grade 2, more than Grade 1 but less than 50% of the cells pyknotic; and Grade 3, more than 50% pyknotic cell bodies in the GCL/INL and ONL.

ImageJ (National Institutes of Health, Bethesda, MD) was used for quantification of TUNEL-labeled cells. On each image, the inner retina (from the INL to the inner limiting membrane) and outer retina (ONL) were delineated using the polygon selection tool, and TUNEL-positive cells were automatically counted using the ImageJ plugin Cell Counter.[28]

PKC-α–labeled images were used to quantify rod bipolar cells. The number of labeled cell bodies in the INL was counted manually. Only well-labeled and whole cell bodies were counted. In addition, total PKC-α expression in rod bipolar cells, including dendrites, cell bodies, axons, and terminals, was measured by using the polygon selection tool in ImageJ including the outer plexiform layer (OPL), INL, and inner plexiform layer (IPL) with subsequent analysis of mean fluorescence intensity. To quantify rod and cone bipolar cells, CHX10-labeled images were used. The number of labeled cells was assessed by cell counting in the INL using the ImageJ plugin Cell Counter.[28] To assess the number of ganglion cells, RBPMS-labeled images were used and labeled cells counted manually. Only completely labeled cells positioned in the ganglion cell layer were counted. Debris and fragmented cellular structures were excluded.

Statistical Analyses

Morphological data from all pressure groups, as well as LDH data from the sampled media, were statistically analyzed. For LDH analysis, the three media samples per explant were pooled, and the average activity (absorbance level) was calculated. For morphological evaluation, calculation of the coefficient of variation (standard deviation/mean) for each staining/labeling from each explant was performed, and it was found to be above 15% in the far majority of instances, indicating a high degree of regional variability. For this reason, raw data obtained from each image were used in these statistical analyses. Prism 9.4.1 for Mac (GraphPad, San Diego, CA) was used for the statistical analyses. For H&E-stained sections, the ordinal pyknosis data were analyzed using the Kruskal–Wallis test with the addition of Dunn's multiple comparisons test. Because the median in all compared groups was the same, the frequency distribution of pyknosis (%) was calculated and presented.

For the remaining morphological analyses, a normality test was performed for data in all groups, using D'Agostino's and Pearson's tests. In cases where the majority of groups in the respective labeling/staining passed the normality test, one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was performed, and the mean and standard deviation (SD) were calculated. In the remaining analyses, the Kruskal–Wallis test with Dunn's multiple comparisons test was used to compare groups, with calculations of the median and interquartile range. For assessment of the dynamic pattern of RBPMS-labeled ganglion cells at each pressure, data were analyzed using the Mann–Whitney test with median and interquartile range. $P < 0.05$ was considered significant.

Results

Overall Tissue Damage

LDH Analysis

The intracellular enzyme LDH, a cell damage marker, was sampled and measured in the culture media to assess the cellular viability in retinal explants. Explants subjected to a pressure of 10 mmHg for 24 hours displayed significantly lower LDH release compared with the remaining pressure groups (0-mmHg, $P < 0.05$; 30-mmHg, $P < 0.01$; 60-mmHg, $P < 0.05$) (Fig. 1). No difference in LDH release was found after 48 hours in culture.

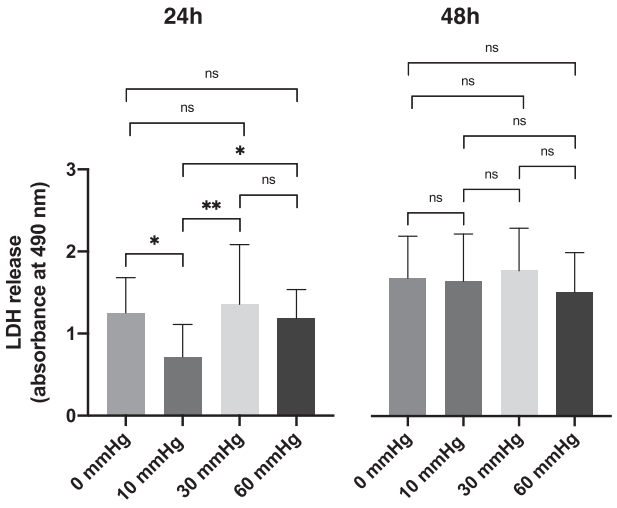

Figure 1. Statistical analysis (one-way ANOVA) of the cell damage marker LDH in culture media derived from retinal explants subjected to a pressure of 0, 10, 30, or 60 mmHg for 24 or 48 hours. After 24 hours in culture, the 10-mmHg group displayed significantly lower LDH release in the media compared with the other pressure groups. After 48 hours in culture, there was no difference among the groups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. *Bars* represent mean values; *error bars*, standard deviations. Pooled data from each explant were used.

H&E Staining

H&E staining was used to assess pyknosis in the inner and outer retina. In directly fixed control retinas, the inner and outer retina displayed only occasional pyknotic cell bodies (Fig. 2A). Retinas in all pressure groups (0, 10, 30, and 60 mmHg) showed pyknotic cell bodies in all three nuclear layers after 24 and 48 hours in culture (Figs. 2B–2I). After 24 and 48 hours in culture, a significantly lower amount of pyknotic cell bodies was found in the 10-mmHg group compared with the 30- and 60-mmHg counterparts in the inner retina and compared with all groups in the outer retina: inner retina at 24 hours, $P < 0.01$ (30-mmHg) and $P < 0.01$ (60-mmHg); outer retina at 24 hours, $P < 0.001$ (0-mmHg), $P < 0.0001$ (30-mmHg), and $P < 0.0001$ (60-mmHg); inner retina at 48 hours, $P < 0.05$ (30-mmHg) and $P < 0.001$ (60-mmHg); outer retina at 48 hours, $P < 0.0001$ (0-mmHg), $P < 0.0001$ (30-mmHg), and $P < 0.0001$ (60-mmHg) (Figs. 2J, 2K).

TUNEL

TUNEL staining was performed to detect apoptotic cells in the inner and outer retina. In directly fixed control retinas, almost no TUNEL-labeled cells were found in the inner and outer retina (Fig. 3A). In contrast, cultured retinal explants from all pressure groups displayed labeled cells with varying intensity in the inner and outer retina (Figs. 3B–3I). After 24 hours in culture, the number of TUNEL-positive cells was significantly lower in retinas subjected to a pressure of 10 mmHg compared with all other pressure groups in the inner retina and compared with the 30- and 60-mmHg groups in the outer retina: inner retina, $P < 0.001$ (0-mmHg), $P < 0.0001$ (30-mmHg), and $P < 0.001$ (60-mmHg); outer retina, $P < 0.001$ (30-mmHg) and $P < 0.01$ (60-mmHg) (Fig. 3J). In addition, a higher number of positive cells was detected in the 30-mmHg group compared with the remaining pressure groups in the inner retina (0-mmHg, $P < 0.001$; 10-mmHg, $P < 0.0001$; 60-mmHg, $P < 0.001$) (Fig. 3J).

After 48 hours in culture, a significantly lower number of TUNEL-positive cells was found in the 10-mmHg group compared with all other pressure groups in the inner and outer retina: inner retina, $P < 0.001$ (0-mmHg), $P < 0.0001$ (30-mmHg), and $P < 0.001$ (60-mmHg); outer retina, $P < 0.0001$ (0-mmHg), $P < 0.0001$ (30-mmHg), and $P < 0.0001$ (60-mmHg) (Fig. 3K). The number of labeled cells in the 30-mmHg group was significantly higher compared with remaining pressure groups in the inner retina (0-mmHg, $P < 0.0001$; 10-mmHg, $P < 0.0001$; 60-mmHg, $P < 0.0001$) (Fig. 3K).

Inner Retinal Neurons

Rod Bipolar Cells—PKC-α Immunohistochemistry

PKC-α immunohistochemistry was used for the detection of rod bipolar cells. Directly fixed control retinas displayed well-labeled rod bipolar cell bodies in the outer part of the INL, vertical axons extending to the IPL, and dendrites in the OPL (Fig. 4A). In these retinas, labeling was present throughout the specimens, whereas in cultured explants PKC-α labeling displayed a varying pattern, with some regions containing well-labeled cells and others almost no labeling (Figs. 4B–4I).

When counting the number of PKC-α–labeled cell bodies after 24 and 48 hours in culture, no difference was found in the 10-mmHg group compared with directly fixed control retinas. A lower number of labeled cells was detected in the 0-, 30-, and 60-mmHg groups compared with the 10-mmHg group (0-mmHg, $P < 0.0001$; 30-mmHg, $P < 0.0001$; 60-mmHg, $P < 0.0001$) (Figs. 4J, 4K). In addition, after 48 hours of culture, a lower number of labeled cells was found in the 60-mmHg group compared with the 0- and 30-mmHg counterparts (0-mmHg, $P < 0.05$; 30-mmHg, $P < 0.05$) (Fig. 4K).

To assess the total expression of PKC-α, the mean fluorescence intensity was measured using ImageJ. After 24 and 48 hours in culture, the 10-mmHg group displayed higher fluorescence intensity compared with

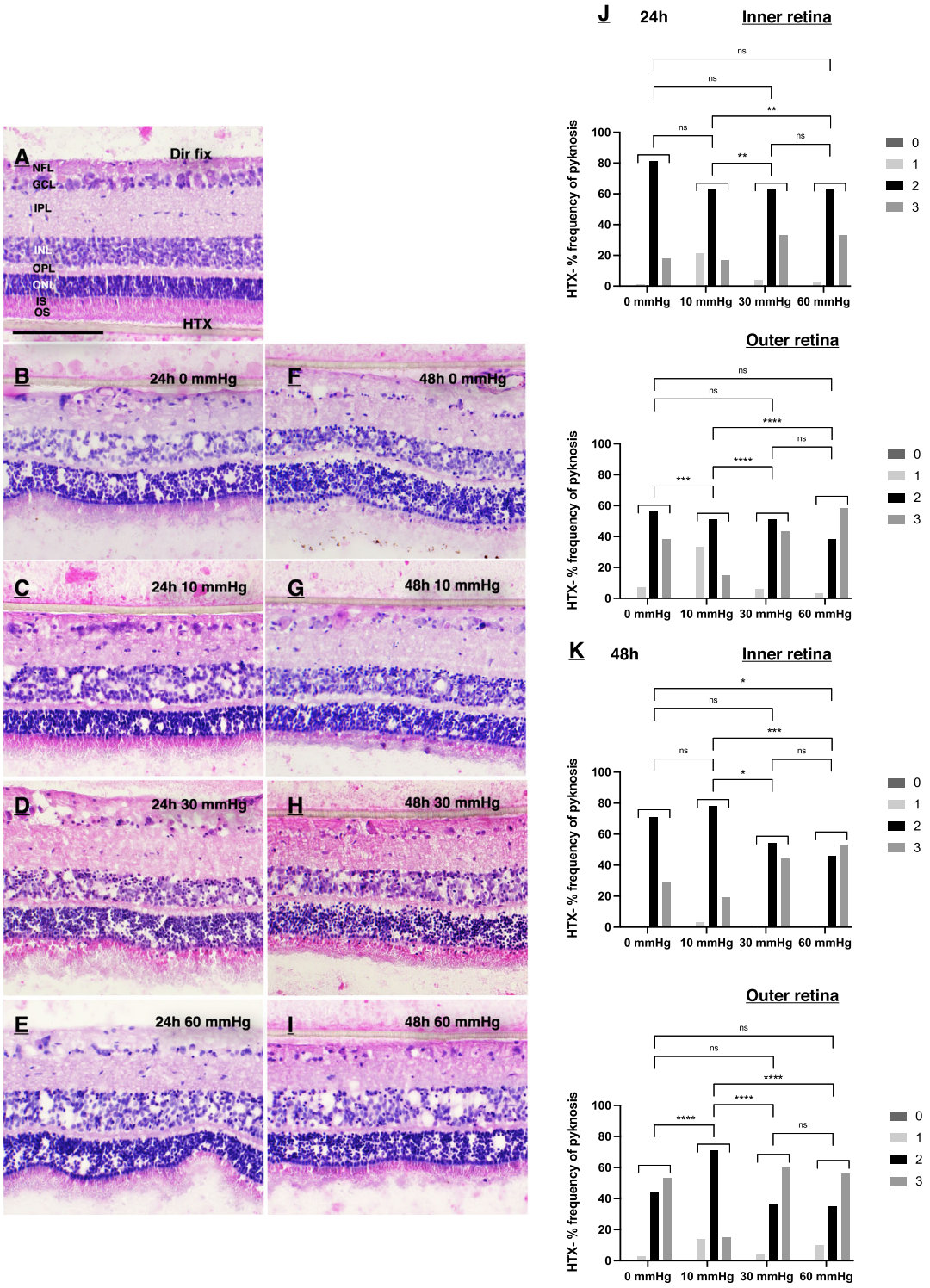

Figure 2. Statistical analysis (Kruskal–Wallis) of the overall tissue damage score using H&E staining for assessment of pyknosis in the inner (INL and GCL) and outer (ONL) retina. Pyknosis was scored in the inner and outer retina separately from 0 to 3 (see Materials and Methods). The frequency distribution of the pyknosis scoring (0–3) is shown in the *bar graphs*. (A) Directly fixed control retina. (B–E) Retinal explants subjected to pressure for 24 hours in culture. (F–I) Retinal explants subjected to pressure for 48 hours in culture. (J, K) After 24 and 48 hours in culture, a significantly lower amount of pyknosis was seen in the 10-mmHg group compared with the 30- and 60-mmHg groups in the inner retina and compared with all pressure groups in the outer retina. *Bars* represent median values; *error bars*, interquartile ranges. Raw data were used for the analyses. OS, outer segment; IS, inner segment; NFL, nerve fiber layer. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. *Scale bar*: 100 µm.

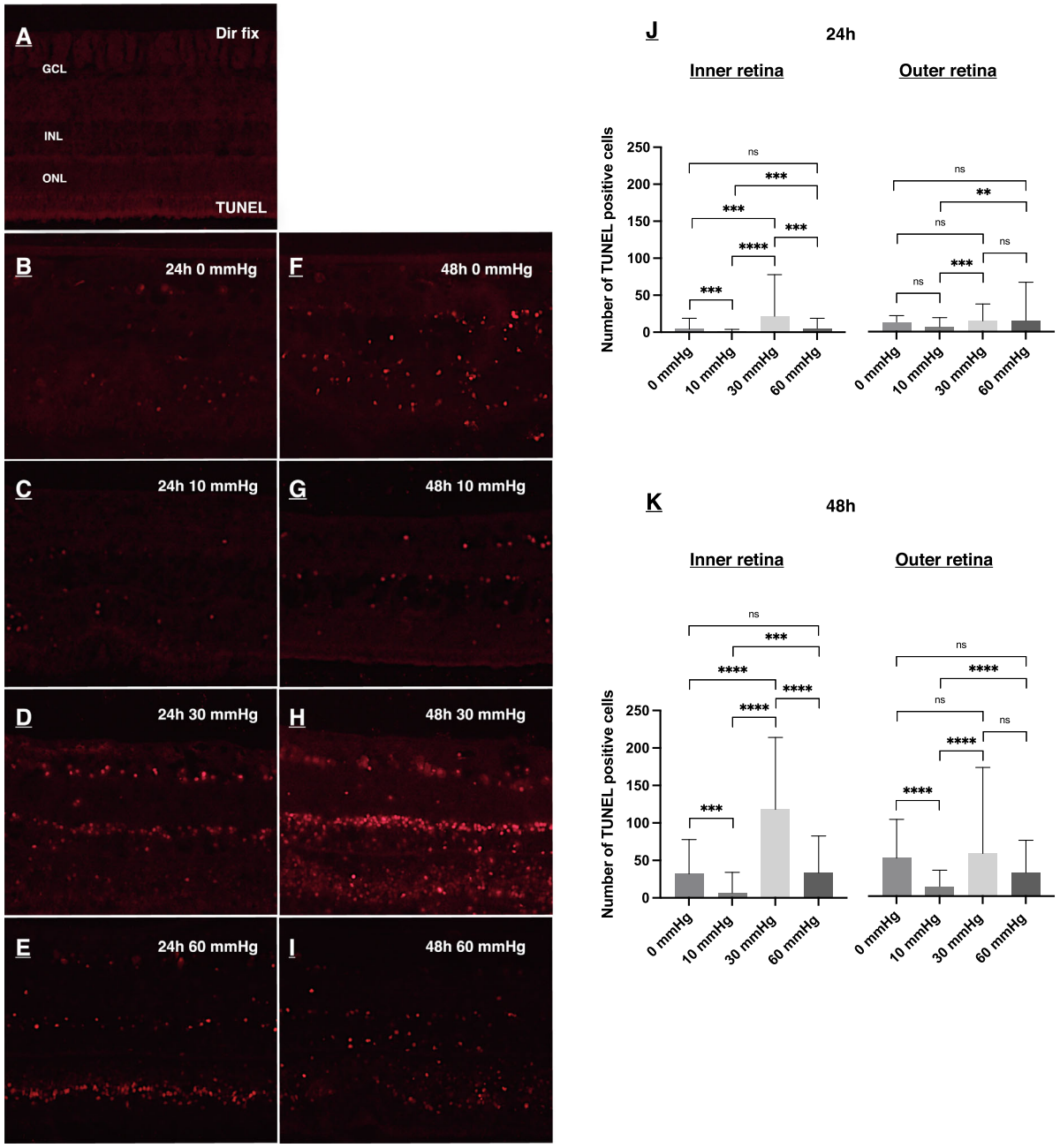

Figure 3. Statistical analysis (Kruskal–Wallis) of TUNEL staining for assessment of apoptotic cells in the inner (GCL and INL) and outer (ONL) retina. (A) Directly fixed control retina. (B–E) Retinal explants subjected to pressure for 24 hours in culture. (F–I) Retinal explants subjected to pressure for 48 hours in culture. (J) After 24 hours in culture, a significantly lower number of labeled cells was found in the 10-mmHg group compared with all pressure groups in the inner retina and compared with the 30- and 60-mmHg counterparts in the outer retina. (K) After 48 hours in culture, the 10-mmHg explants showed significantly fewer labeled cell bodies compared with the remaining pressure groups in the inner and outer retina. *Bars* represent median values; *error bars*, interquartile ranges. Raw data were used for the analyses. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. *Scale bar*: 100 µm.

all other groups: at 24 hours, $P < 0.001$ (directly fixed control retinas), $P < 0.0001$ (0-mmHg), $P < 0.0001$ (30-mmHg), and $P < 0.0001$ (60-mmHg); at 48 hours, $P < 0.01$ (directly fixed control retinas), $P < 0.0001$ (0-mmHg), $P < 0.0001$ (30-mmHg), and $P < 0.0001$ (60-mmHg) (Figs. 4L, 4M). Cultured retinas from remaining pressure groups (0-, 30-, and 60-mmHg) displayed no significant difference in fluorescence intensity when compared with directly fixed control explants. The 60-mmHg explants displayed significantly lower intensity compared with all other cultured groups (for P values) (Figs. 4L, 4M).

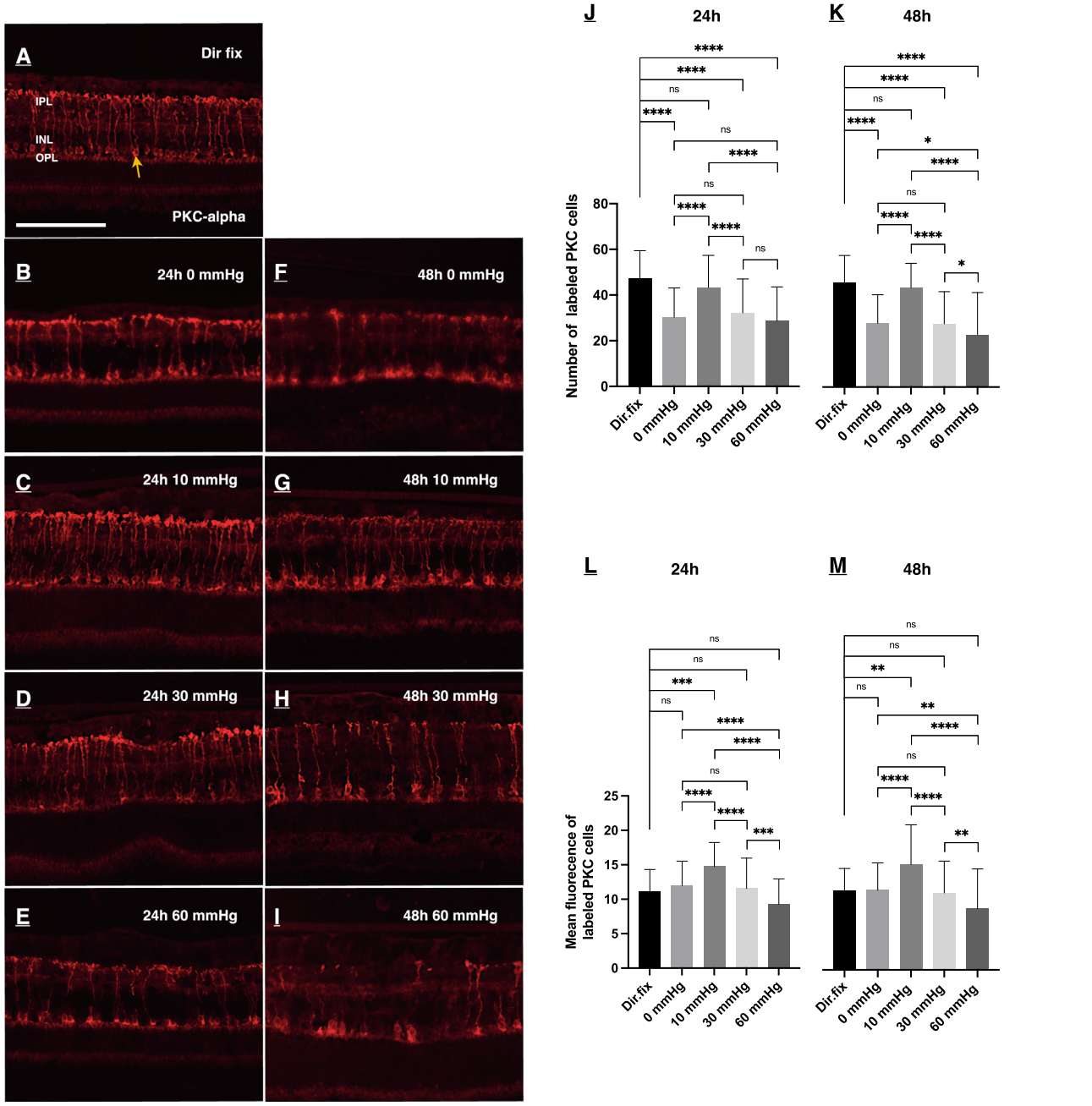

Figure 4. Statistical analysis (one-way ANOVA) of PKC-α labeling for assessment of rod bipolar cells. (A) Directly fixed control retina with cell bodies in the outer INL (*arrow*). (B–E) Retinal explants subjected to pressure for 24 hours in culture. (F–I) Retinal explants subjected to pressure for 48 hours in culture. (J, K) After 24 and 48 hours in culture, a significantly higher number of labeled cells was found in retinas subjected to 10 mmHg compared with the remaining pressure groups. (L, M) After 24 and 48 hours in culture, significantly higher fluorescence intensity was seen in the 10-mmHg group compared with all groups. *Bars* represent mean values; *error bars*, standard deviations. Raw data were used for the analyses. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. *Scale bar*: 100 µm.

Cone and Rod Bipolar Cells—CHX10 Immunohistochemistry

CHX10 immunohistochemistry was used for the detection of cone and rod bipolar cells. In directly fixed control retinas, CHX10-labeled sections revealed several rows with well-labeled, small, and round cell bodies situated in the outer part of the INL (Fig. 5A). The cultured retinal specimens also displayed well-labeled cells in the outer part of the INL; however, areas with little or almost no labeling were also found (Figs. 5B–5I). After 24 hours of culture, no significant difference in the number

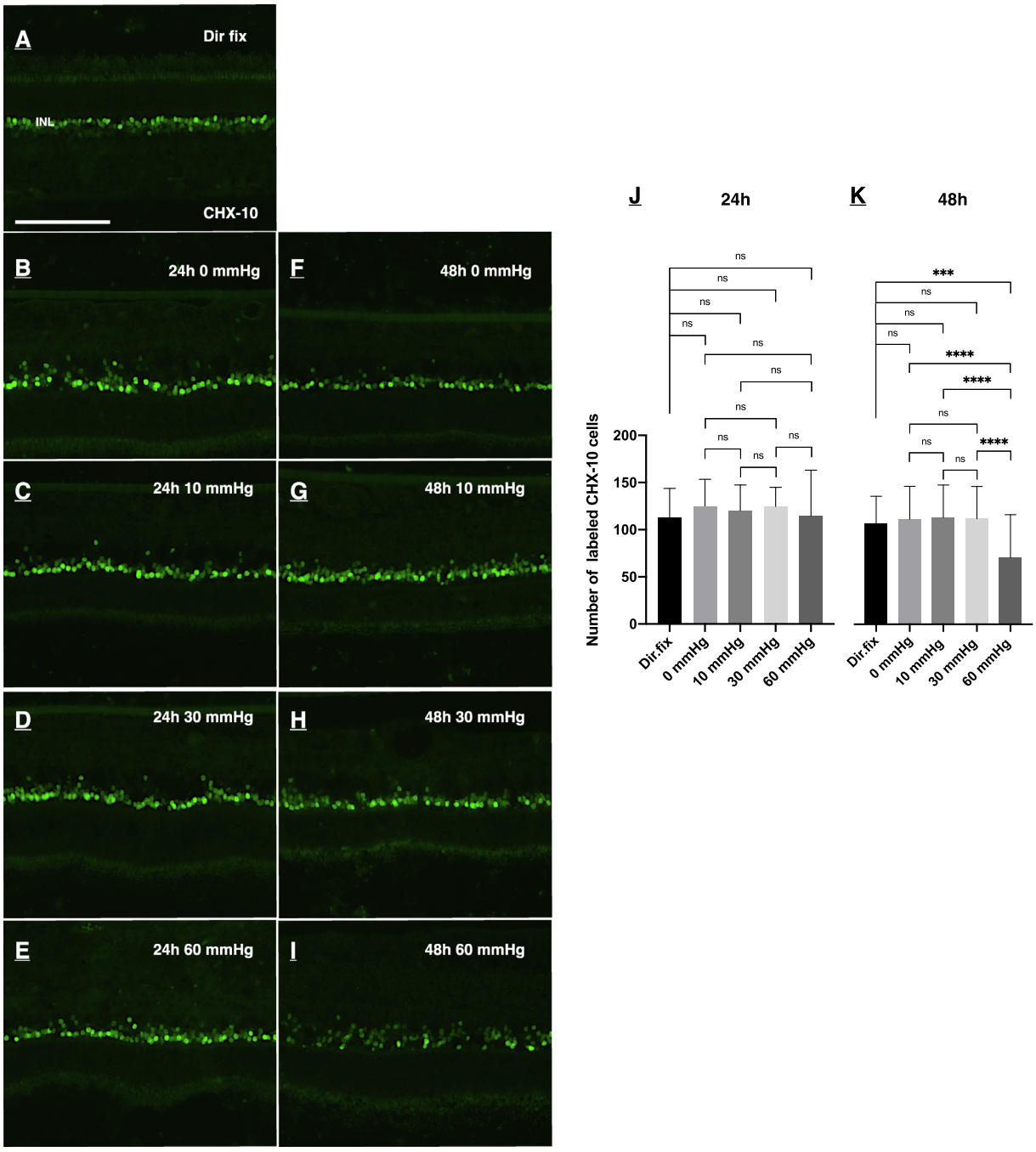

Figure 5. Statistical analysis (one-way ANOVA) of CHX10 labeling for assessment of rod and cone bipolar cells. (A) Directly fixed control retinas. (B–E) Retinal explants subjected to pressure for 24 hours in culture. (F–I) Retinal explants subjected to pressure for 48 hours in culture. (J) In retinal explants cultured for 24 hours, no difference in the number of labeled cells was found among the groups. (K) In retinal explants cultured for 48 hours, a significantly lower number of labeled bipolar cells in the 60-mmHg group was seen compared with the remaining groups. *Bars* represent mean values; *error bars*, standard deviations. Raw data were used for the analyses. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. *Scale bar*: 100 µm.

of labeled cells was detected among any of the groups. After 48 hours in culture, a lower number of cells was found in the 60-mmHg group compared with all other groups (directly fixed control retinas, $P < 0.001$; 0-mmHg, $P < 0.0001$; 10-mmHg, $P < 0.0001$; 30-mmHg, $P < 0.0001$) (Fig. 5K).

Amacrine Cells—Parvalbumin Immunohistochemistry

Parvalbumin immunohistochemistry was performed for detection of amacrine cells in the INL. Parvalbumin labeled sections in directly fixed controls and cultured explants revealed well-labeled, round cell bodies in the innermost INL and GCL, and two

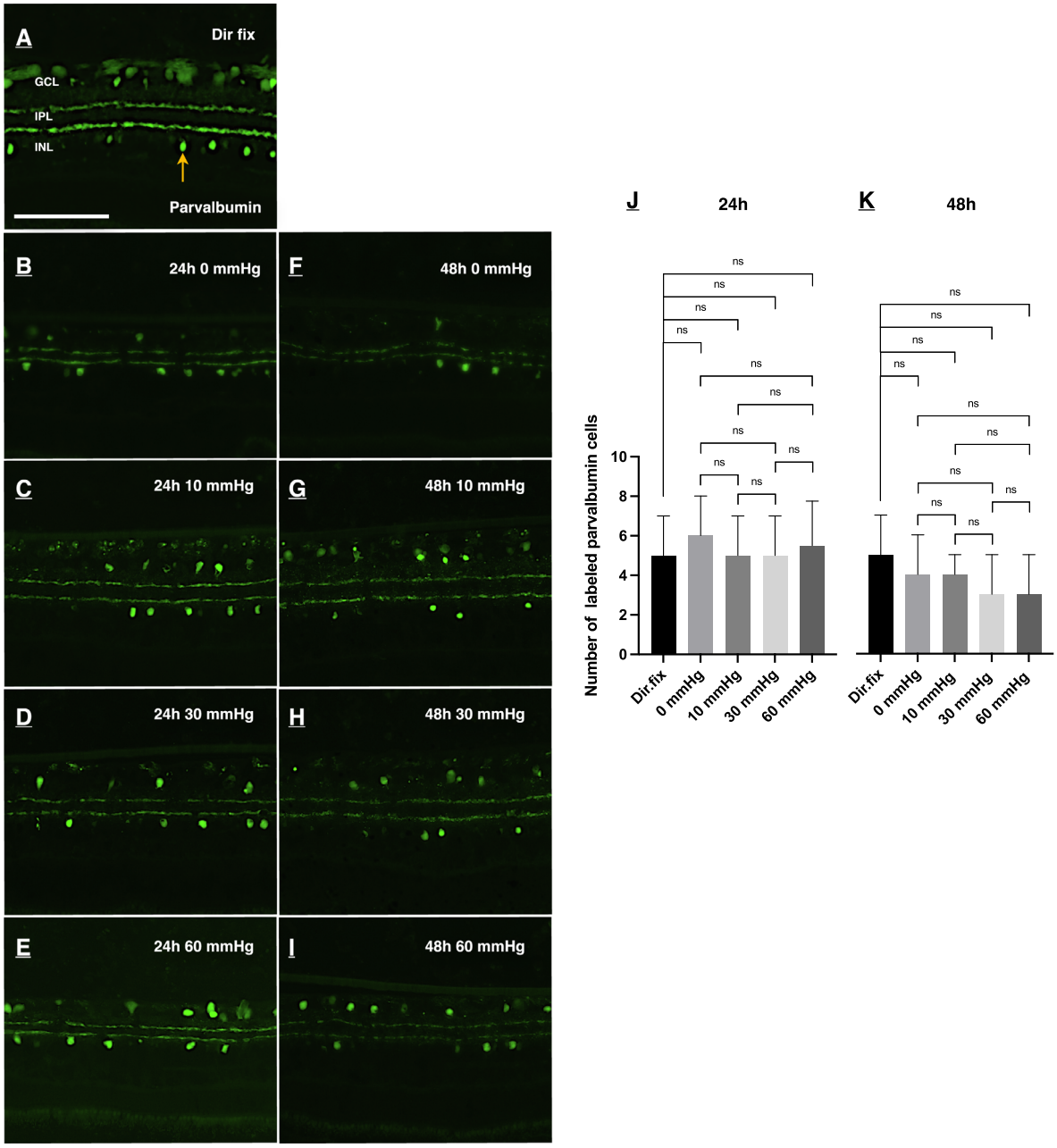

Figure 6. Statistical analysis (Kruskal–Wallis) of parvalbumin labeling for assessment of amacrine cells in the INL. (A) Directly fixed control retinas, with amacrine cell bodies in the innermost INL (*arrow*) and in the GCL (not counted). (B–E) Retinal explants subjected to pressure for 24 hours in culture. (F–I) Retinal explants subjected to pressure for 48 hours in culture. (J, K) After 24 and 48 hours in culture, no difference in the number of cells was found in any of the groups. *Bars* represent median values; *error bars*, interquartile ranges. Raw data were used for the analyses. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. *Scale bar*: 100 µm.

labeled horizontal strata in the IPL (Figs. 6A–6I). For cell counts, cells in the GCL were excluded, and only well-labeled and round cells in the innermost INL were counted. After 24 and 48 hours in culture, no difference in the number of labeled cells was found in any of the groups.

Ganglion Cells—RBPMS Immunohistochemistry

RBPMS immunohistochemistry was performed for the detection of ganglion cells. RBPMS-labeled sections in directly fixed control retinas and cultured retinas displayed well-labeled and mainly large cell bodies in the GCL; however, in cultured explants,

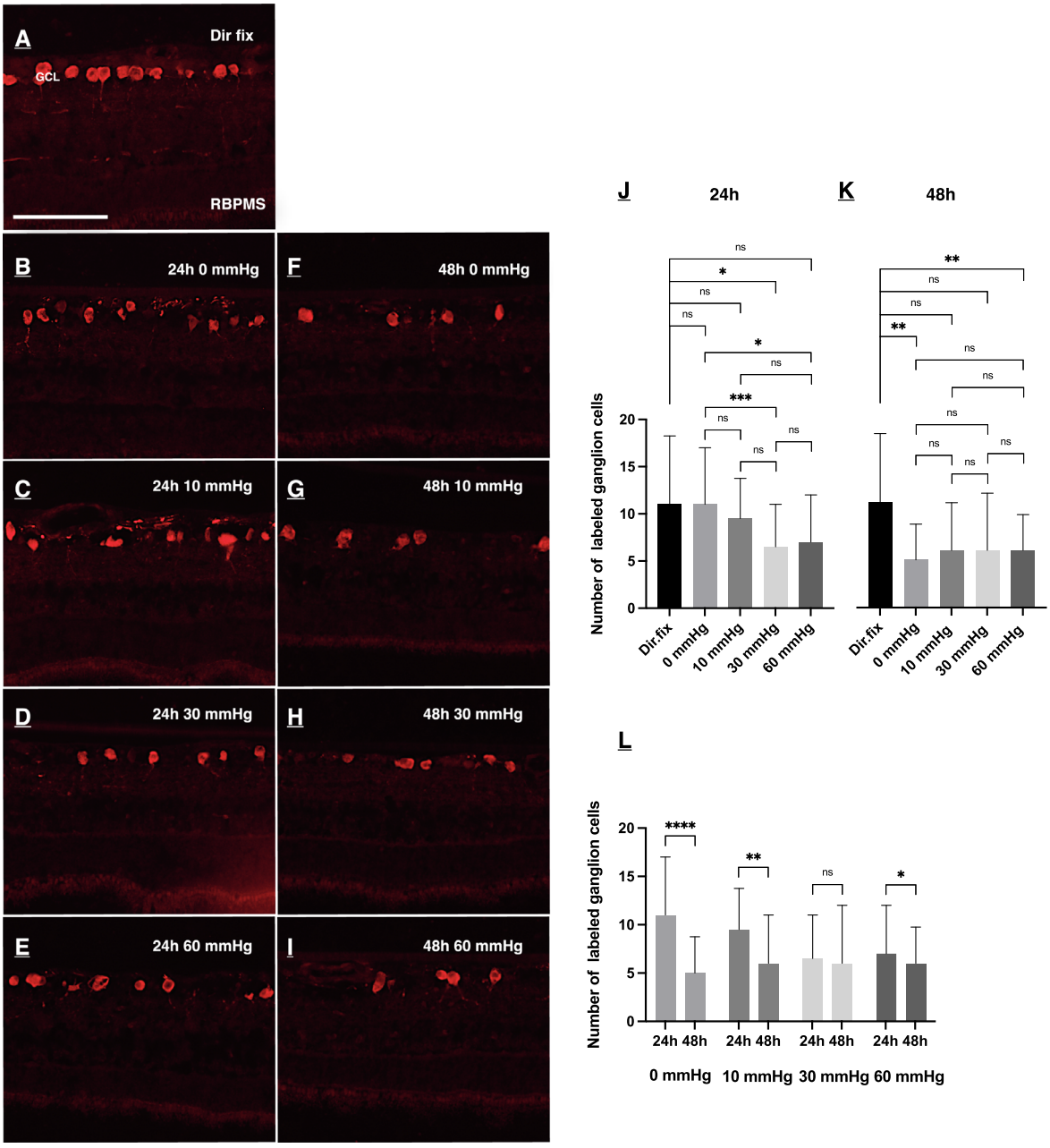

Figure 7. Statistical analysis (Kruskal–Wallis) of RBPMS labeling for assessment of ganglion cells in the GCL. (A) Directly fixed control retina. (B–E) Retinal explants subjected to pressure for 24 hours in culture. (F–I) Retinal explants subjected to pressure for 48 hours in culture. (J) After 24 hours in culture, a lower number of labeled cells was seen in the 30-mmHg group compared with directly fixed control retinas. (K) After 48 hours in culture, a lower number of labeled cells was found in the 0- and 60-mmHg groups compared with directly fixed control retinas; no differences were detected among the pressure groups. (L) Statistical analysis (Mann–Whitney) comparing the number of RBPMS-labeled cells in each pressure group at 24 and 48 hours in culture showed a decrease in the 0-, 10-, and 60-mmHg groups after 48 hours in culture. *Bars* represent median values; *error bars*, interquartile ranges. Raw data were used for the analyses. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. *Scale bar*: 100 µm.

fragmented structures were also seen (Figs. 7A–7I). In retinas cultured for 24 hours, no differences in the number of RBPMS-labeled ganglion cells were detected between the directly fixed control retinas compared with the 0-, 10-, and 60-mmHg groups, whereas a significantly lower number of cells was seen in the 30-mmHg counterpart ($P < 0.05$) (Fig. 7J). There was no difference in the number of RBPMS-labeled cells among the 10-, 30-, and 60-mmHg groups; however, a significantly higher number of labeled cells

was seen in the 0-mmHg group compared with the 30- and 60-mmHg counterparts (30-mmHg, $P < 0.001$; 60-mmHg, $P < 0.05$) (Fig. 7J). After 48 hours of culture, a lower number of labeled cells was detected in the 0- and 60-mmHg groups compared with directly fixed control retinas (0-mmHg, $P < 0.01$; 60-mmHg, $P < 0.01$) (Fig. 7K). No differences were detected when comparing the pressure groups. To assess the dynamic pattern of RBPMS-labeled ganglion cells at each pressure, a comparison of labeled cells at 24 and 48 hours was performed separately (Fig. 7L). Compared to 24 hours in culture, a significant decrease in the number of labeled cells was found in the 0-, 10-, and 60-mmHg groups after 48 hours in culture (0-mmHg, $P < 0.0001$; 10-mmHg, $P < 0.01$; 60-mmHg, $P < 0.05$) (Fig. 7L). In the 30-mmHg group, no such decrease was detected.

Discussion

Summary

In this paper, we wanted to explore pressure-related effects on the retina in a large-animal model, with the aim of investigating any possible beneficial as well as detrimental impacts. We concentrated our efforts on overall tissue damage and morphological changes in the principal inner retinal neurons: bipolar, amacrine, and ganglion cells. To this end, we used an in vitro system in which the pressure causing compression of the retina can be precisely controlled and isolated without the influence of other mechanical stimuli such as tissue stretch. By supplanting the tissue with nutrition and oxygen from the media, the influence of ischemia is also minimized. A disadvantage of using compressed air to increase pressure is the possibility of pH and O_2 changes in the media.[29] Osborne et al.,[15] in a similar experiment using human retinal explants, showed no change in pH between cultures kept under 0 mmHg (atmospheric pressure) compared with 60 mmHg, whereas oxygen partial pressure was slightly increased.[15]

We chose to compare cultured retinal explants for 24 and 48 hours with the pressure set at 0 mmHg (atmospheric pressure only), 10 mmHg to resemble normal IOP, 30 mmHg to correspond to conditions in primary open-angle glaucoma, and 60 mmHg to duplicate acute glaucoma. Interestingly, we found that retinas subjected to a moderate amount of pressure (10 mmHg) showed less overall tissue damage and superior preservation of rod bipolar cell morphology compared with retinas in which no pressure was applied. This finding has, to our knowledge, not been described previously. We also found that excessive pressure (30 and 60 mmHg) resulted in increased tissue damage and impaired rod bipolar morphology after 24 hours.

Moderate Pressure is Beneficial for Retinal Homeostasis

The interior of the eye constitutes a highly biomechanical environment in which retinal cells are constantly exposed to IOP. The retina has been found to be mechanosensitive, responding to dynamic forces such as shear stress, mechanical strain, and tension from the nearby environment.[1,3] We have previously found that adult porcine retinal explants subjected to lateral tension survive better in the in vitro milieu compared with tissue kept with no such tension.[30] With this in mind, comparing retinas cultured within the pressure chamber at 0 mmHg with retinas subjected to moderate pressure (10 mmHg) is especially interesting.

Previous studies have shown that the axotomy and loss of contact with the retinal pigment epithelium (RPE), as well as ischemia during the time from enucleation to actual explantation included in the culture paradigm, invariably results in degeneration of the adult retina in vitro.[31–33] To improve retinal survival, we used an optimized in vitro model and cultured the retinal explants with the inner limiting membrane (ILM) facing the culture membrane, which has been shown to attenuate culture-related degeneration.[22] We also ensured that explantation was performed within 90 minutes after enucleation, which also limits tissue damage.[33] In spite of these efforts, evidence of culture-related damage was clearly present in retinas cultured under atmospheric pressure (0 mmHg).

In contrast to retinas cultured at 0 mmHg, several of our findings indicate that a pressure of 10 mmHg attenuated culture-related neuronal degeneration, suggesting that this moderate pressure was in fact beneficial for retinal homeostasis. The LDH analysis, an objective method for investigation of cellular damage, showed that LDH release in media derived from explants subjected to 10 mmHg was significantly lower compared to all pressure counterparts, including the 0-mmHg group, after 24 hours in culture. Further, the 10-mmHg group displayed a lower amount of pyknosis and apoptosis after 24 and 48 hours in culture. Finally, using PKC-α labeling, no difference in the number of rod bipolar cell bodies was detected in the 10-mmHg group compared with directly fixed control retinas at both time points, whereas the number of cells was significant lower in the 0-mmHg group.

The reason why the interior of the normal eye is constantly subjected to the IOP has not been elucidated. Similarly, why 10-mmHg explants in our

study showed improved survival compared to the 0-mmHg counterparts is not readily apparent. Various mechanosensitive channels responding to a range of stimuli including pressure have been found in retinal neurons, as well as in glial cells.[1,34] These channels interact with the extracellular matrix and the cytoskeleton, resulting in profound effects on retinal homeostasis, including calcium ion (Ca^{2+}) transport.[1,3] Thus, an intricate system of mechanotransduction exists in the retina, and our results indicate that stimulation of this system by a moderate amount of pressure may be important for maintaining retinal neurons and tissue homeostasis within the culture environment.

Interestingly, when assessing the total expression of PKC-α using ImageJ, the mean fluorescence intensity was found to be significantly higher in the 10-mmHg group compared with directly fixed controls, whereas no difference was found in the 30- and 60-mmHg groups compared with these controls. Protein kinase C is a group of enzymes highly expressed in neurons and vital for various cellular functions. In the retina, the PKC-α isoform is specifically expressed in rod bipolar cells and is involved in phototransduction and inner retinal signal transmission.[35] The PKC-α enzyme can be activated by a range of stimuli and upon such activation translocate from the soma to the axon terminals in the IPL.[35] The finding of increased rod bipolar cell survival combined with an increased overall PKC-α expression in 10-mmHg explants suggests that the enzyme may be an important player involved in the retinal tissue response to pressure.

Excessive Pressure Effects on Inner Retinal Neuronal Subtypes

Excessive IOP is a well-known risk factor for retinal neuronal death in glaucoma. Detrimental pressure-related effects in the form of progressive ganglion cell death have been well studied in several in vivo and in vitro glaucoma models.[6,7,16,36] The prime focus of earlier studies has been on pressure-related ganglion cell death; however, rod bipolar cells and amacrine cells have previously been shown to undergo early changes in the form of functional impairment and morphological changes when subjected to elevated pressure.[10–13] We found that retinas subjected to 30 and 60 mmHg displayed a higher amount of pyknosis and apoptosis compared with retinas subjected to a pressure of 10 mmHg after 24 and 48 hours in culture, and there was greater LDH release in the media after 24 hours. This suggests that a significant number of retinal cells are affected by excessive pressure in vitro.

In our experiment, ganglion cells did not appear to be especially affected by excessive pressure. Compared with directly fixed control retinas, fewer ganglion cells were found in the 30-mmHg group after 24 hours and 60-mmHg group after 48 hours in culture. However, the 0-mmHg group also showed fewer ganglion cells after 48 hours. This is in line with the study by Osborne et al.,[15] who did not find any relationship between excessive pressure and ganglion cell death in a similar setup. Given the particular interest in pressure-related effects on ganglion cells, we also chose to assess the dynamic pattern of RBPMS-labeled ganglion cells in the pressure groups. After 48 hours in culture, a decrease in the number of labeled cells was found in the 0-, 10-, and 60-mmHg groups compared with the 24-hour counterparts, indicating that excessive pressure may not be the cause of ganglion cell degeneration within the pressure culture model. Progressive loss of ganglion cells after axotomy of the optic nerve (which is a part of the explant paradigm) is a well-known event and most likely explains the decline of ganglion cells after 48 hours.[31]

Interestingly, we found no loss of parvalbumin-labeled amacrine cells in the pressure groups compared with directly fixed retinal explants at either 24 or 48 hours in culture, and no differences were detected when comparing the pressure groups. This suggests that culture-related degeneration and excessive pressure effects are not general phenomena and strengthens the notion that these factors affect specific retinal neuronal subtypes.

In contrast, the number of labeled rod bipolar cells was distinctly lower in explants subjected to 30 and 60 mmHg after 24 hours compared with directly fixed controls, which was not the case for the 10-mmHg counterparts. When examining the entire bipolar cell population (rod and cone bipolars), a lower number of CHX10-labeled cells was found at 48 hours but not at 24 hours, and only in the 60-mmHg group, indicating that cone bipolar cells may be less sensitive to pressure-related damage compared with rod bipolar counterparts.

Relevance for Glaucoma Research

Historically, glaucoma has been considered a disease related to elevated IOP.[37] Two main theories have been considered related to the neuropathy seen in glaucoma: direct mechanical disturbance of the tissue and insufficient blood supply (ischemia).[38] Currently, glaucoma is often classified as either open-angle with an IOP within normal limits or angle-closure with an elevated IOP.[4] Although the relevance of the IOP in glaucoma pathogenesis has decreased, lowering the IOP remains the only treatment and has been shown to slow the progression of the disease, even in patients with relatively low IOP.[39]

Previous glaucoma studies have mainly focused on the loss of retinal ganglion cell axons in the nerve fiber layer; however, pathological changes have also been described in the INL. Clinically, a correlation between increased thickening of the INL and the severity of glaucomatous functional loss in patients has been shown by using spectral-domain optical coherence tomography,[13] and neuronal subtypes within this layer have been implicated in primary glaucoma-related damage. In our model, we isolated the compressive pressure component from ischemia and found a lower number of PKC-labeled rod bipolar cells already after 24 hours in culture when subjected to excessive pressure, suggesting a relationship to compressive force. As mentioned above, this was not the case for ganglion or amacrine cells. In line with this finding, rod bipolar cells have been shown to undergo functional loss prior to ganglion cells during elevated pressure.[12] In addition, Shen et al.,[40] using three different models, including ocular hypertension, showed that the PKC-α enzyme and rod bipolar response sensitivity were altered before ganglion cells. Even though the exact role of rod bipolar cells and the PKC-α enzyme in glaucoma is still not elucidated, our findings combined with earlier work suggest a role of rod bipolar cells and the PKC enzyme in early glaucoma pathology.

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