The porcine iodoacetic acid model of retinal degeneration: morpho-functional characterization of the visual system.

Francesca Barone[1,2,¶], Luisa Vera Muscatello[3,¶], Domenico Ventrella[3,*], Alberto Elmi[3], Noemi Romagnoli[3], Luciana Mandrioli[3], Josè Fernando Maya-Vetencourt[4,5], Cristiano Bombardi[3], Maurizio Mete[2], Giuseppe Sarli[3], Fabio Benfenati[4,6], Grazia Pertile[2], Maria Laura Bacci[3]

[1] National Eye Institute, National Institutes of Health, 10 Center Dr, Bethesda, 20814 (MD) US

[2] Ophthalmology Department, Sacro Cuore Hospital - Don Calabria, Via Don A. Sempreboni 5, Negrar, 37024 (VR) IT

[3] Department of Veterinary Medical Sciences, Alma Mater Studiorum Univerisity of Bologna, Via Tolara di Sopra 50, Ozzano dell'Emilia, 40064 (BO) IT

[4] Center for Synaptic Neuroscience and Technology, Italian Institute of Technology, Via Morego 30, Genova, 16163 (GE) IT

[5] Department of Biology, University of Pisa, Via Alessandro Volta 4Bis, Pisa, 56126 (PI) IT

[6] Department of Experimental Medicine, University of Genoa, Via Leon Battista Alberti 2, Genova, 16132 (GE) IT

* **corresponding author**: domenico.ventrella2@unibo.it; ORCID: 0000-0002-9330-979X

¶ These authors equally contributed to the present work

Abstract

Porcine models of ophthalmological diseases are often used in pre-clinical translational studies due to pigs' similarities to humans. In particular, the iodoacetic acid (IAA) model of photoreceptor degeneration seems to mimic well the endstage phenotype of human pathologies as retinitis pigmentosa and age-related macular degeneration, with high potential for prosthesis/retinal devices testing. IAA is capable of inducing photoreceptor death by blockage of glycolysis, and its effects on the retina have been described. Nonetheless, up to date, literature lacks of a comprehensive morpho-functional characterization of the entire visual system of this model. This gap is particularly critical for prosthesis testing as inner retinal structures and optic pathways must be preserved to elicit cortical responses and restore vision. In this study, we investigated the functional and anatomical features of the visual system of IAA-treated pigs and compared them to control animals. IAA was administered intravenously at 12 mg/kg; control animals received saline solution (NaCl 0.9% w/v). Electrophysiological analyses included full-field (ffERGs) and pattern (PERGs) electroretinograms and flash visually evoked potentials (fVEPs). Histological evaluations were performed on the retina and the optic pathways and included thickness of the different retinal layers, ganglion cells count, and immunohistochemistry for microglial cells, macroglial cells, and oligodendrocytes. The histological results indicate that IAA treatment does not affect the morphology of the inner retina and optic pathways. Electrophysiology confirms the selective rod and partial cone degeneration, but is ambiguous as to the functionality of the optic pathways, seemingly preserved as indicated by the still detectable fVEPs. Overall, the work ameliorates the characterization of such rapid and cost-effective model, providing more strength and reliability for future pre-clinical translational trials.

Keywords: Porcine model; Photoreceptor degeneration; Iodoacetic acid; Visual System; Electrophysiology; Histology.

1 Introduction

Retinal degenerative pathologies, including amongst the others retinitis pigmentosa (RP) and age-related macular degeneration (AMD), are accountable for the majority of blindness and visual impairment cases, with more than 30 million people affected worldwide (Ben M'Barek et al., 2019). Various animal models have been used to study genotype-phenotype correlation and evaluate new therapeutic strategies. Small animals are very useful for an early phase of investigation, but larger animals are very important to establish efficacy and safety parameters prior to human trials (Chader, 2002).

The pig is an excellent model for the human eye and retina; the swine eye has an overall anatomy comparable to the human eye (Prince and Ruskell, 1960) and the retina contains a cone-dominant central visual streak with rods enriched in the peripheral retina (Chandler et al., 1999; Hendrickson and Hicks, 2002; Kostic and Arsenijevic, 2016). Size similarities with the human eye, despite being obvious in comparison to rodents and other small laboratory animals, are challenging to analyze since highly dependent on age and breed of the pig (Middleton, 2010). This features make the pig model an excellent model for testing various therapeutic strategies for photoreceptor degeneration, such as gene therapy, stem cell therapies or implantation of retinal prosthesis (Bertschinger et al., 2008; Manfredi et al., 2013; Sharma et al., 2019). Transgenic pig model have been developed for studying cone degeneration (Petters et al., 1997; Ross et al., 2012; Sommer et al., 2011; Colella et al., 2014). Despite clear advantages, these models share slow time course of disease progression and can only feature a specific gene defect, while photoreceptor degeneration is often a multifactorial disease. This could be a deterrent for their use in cases when the use of a rapidly inducible swine model of photoreceptor damage would be advantageous.

Iodoacetic acid (IAA) was proven to block glycolysis and be toxic to neurons (Orzalesi et al., 1970; Winkler et al., 2003; Liang et al., 2008) and therefore was used to induce photoreceptor degeneration in different species (Farber et al., 1983; Liang et al., 2008; Nan et al., 2013; Rösch et al., 2015). The Iodoacetic acid (IAA) pig model, a rapidly and cost effective model of photoreceptor degeneration, was previously described and characterized (Scott et al., 2011; Wang et al., 2011; Noel et al., 2012) and could be the gold standard in preclinical trials for retinal prosthetics. Retinal prosthesis are rapidly emerging as a therapeutic strategy for photoreceptor degeneration in those situations when other approaches such as gene therapy and stem cell transplant, cannot be applied (Bertschinger et al., 2008; Maya-Vetencourt et al., 2017). Although, in principle, retinal prosthesis can be effective also when photoreceptors are completely degenerated, inner retinal structures and

optic pathways must be preserved to elicit cortical responses and restore vision. It is indeed of high importance to carefully verify the functional and morphological status of the optic pathways in preclinical models.

Although the IAA pig model was previously characterized, the functional and morphological description of the primary optic pathways after its administration was never evaluated and the lack of this information can lead to additional bias and weakness in the translational value of the model. The aim of this study is to morphologically and physiologically characterize the retina and optic pathways in an IAA pig model of end-stage photoreceptors degeneration, from the outer retina to the visual cortex.

2 Materials and Methods

2.1 Animals and study design

The use of animals in this study was regulated by two protocols approved by the Italian Ministry of Health, one with D.Lgs 116/92 and one with the new law D.Lgs 26/2014, in accordance with the Association for Research in Visual Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The procedures described in this paper are part of the phase-one of a bigger project that aimed to describe new therapeutic approaches to restore vision in a pig model of photoreceptor degeneration.

Sixteen commercial hybrid pigs [(Large White x Landrance) x Duroc] (7 neutered males NM and 9 females F, 32 ± 12 Kg bodyweight, between 80 and 90 days old) were enrolled in the study and housed in multiple boxes according to their origin dominance group to reduce stress and aggressiveness. Animals were fed with swine standard diet (CESAC s.c.a. Conselice RA, Italy) twice a day and received water *ad libitum,* room temperature was set a 20 ± 4 °C with a 12/12 hours light/dark cicle with a minimum of 40 lux and a maximum of 80 lux during light period (Barone et al., 2018). Chains and pieces of wood were used as environmental enrichment (Nannoni et al., 2016), no bedding materials were used to guarantee good sanitary conditions, but every two days the animals received a small amount of straw as rooting material.

The animals were anesthetized, and a group was treated with IAA (IAA group n=11, 5 NM and 6 F), while the other group was injected with saline (NaCl 0.9% w/v; CTR group n=5, 2 NM and 3 F), both intravenously. A week before (T0) and a month after (T30) IAA treatment, animals were evaluated with a panel of electrophysiological tests: ffERG, PERG and fVEP. During functional tests, at T0 and T30, blood was sampled for hematologic and biochemical analyses. At the end of the trial,

6 pigs, 3 IAA and 3 CTR F, underwent general anesthesia and were euthanized with an intravenous injection of 0.3 ml/kg of embutramide 200 mg/ml, mebenzonio iodide 50 mg/ml, tetracaine hydrochloride 5 mg/ml (Tanax, Intervet Italia srl, Milan, Italy). The animals were then submitted to necroscopic and histologic investigations. In all subjects, histology focused on the study of the optic tracts and retina samples by light microscopy. The other animals (IAA group n=8, CTR group n=2) underwent the phase-two of the experimentation aiming at studying new therapeutic approaches for the cure of end stage photoreceptor degeneration.

2.2 Anesthesia protocol and IAA treatment

The same anesthetic protocol was used for the electrophysiological analysis and the IAA treatment. Animals were weight and sedated with an intramuscular (IM) injection of Tiletamine-Zolazepam (Zoletil, Virbac, Prague) (5 mg/Kg). After 15 min (15±3 min), general anesthesia was induced with 8 % sevoflurane (SevoFlo, Esteve, Barcelona, Spain), administered through a mask in a 1:1 oxygen/air mixture and maintained with the same halogenated agent (3±0.5 %) after oro-tracheal intubation. Lactated Ringer infusion was set at 10 ml/kg/h rate through the auricular vein (left ear). During anesthesia, hematologic and biochemical analyses were performed. Briefly, samples were obtained from the femoral artery using a 21 G butterfly needle and a vacuum system; tubes with K3EDTA anticoagulant and clot activator were used (Terumo, Leuven, Belgium) (Ventrella et al., 2017). The IAA group piglets received an intravenous injection, in the auricular vein (right ear), of 12 mg/kg body weight of sterile, pH balanced, iodoacetic acid (ICH_2CO_2H) lot n. BCBQ7348V and BCBS2675V (CAS Number: 64-69-7, Sigma Chemical Corp., St Louis, MO) over 5 min. The IAA was freshly prepared on the day of the administration with normal saline (NaCl 0.9% w/v) at the concentration of 12 mg/ml. The animals were maintained under general anesthesia for 30 min after the infusion. Heart frequency rate, non-invasive blood pressure (NIBP), peripheral capillary oxygen saturation SpO_2 and CO_2 were strictly monitored during the post IAA period.

2.3 Electrophysiological analysis

2.3.1 Animal preparation

The analysis was performed at T0 and T30, with the animals under general anesthesia induced with the same protocol described above. Two drops of oxybuprocaine hydrochloride (Novesina, Visufarma s.r.l. Rome, Italy) were used as a local anesthetic, then a barraquer blepharostat was used and the eyes were maintained in a central and stable position by two stay sutures. Corneal contact lenses electrodes (ERG-jet®, Universo Plastique, Switzerland) were used as active electrodes while dermal needle electrodes were used as reference, under the ipsilateral eyelid, and ground, in the

middle of the snout, during ffERG and PERG (Fig. S1A). A drop of 3% carbomer (Dacriogel, Alcon, Fort Worth, Texas, USA) was applied to the inner surface of the lens electrode to protect the cornea and to ensure good electrical contact. For fVEP, a dermal needle was placed close to the occipital protuberance as active electrode (Fig. S1B).

2.3.2 Electrophysiology protocol

The International Society for Clinical Electrophysiology of Vision (ISCEV) Standards for ffERGs, PERG and fVEP (Bach et al., 2013; McCulloch et al., 2015; Odom et al., 2016) were used with the settings adjusted for the swine species, as displayed in Table 1. The ffERG stimuli (Light-adapted 3.0 ERG, Dark-adapted 0.01 ERG, Dark-adapted 3.0 ERG) were produced by a Ganzfeld dome and the patterned stimuli by a screen connected to a pattern generator. The data were amplified and acquired by WinAverager Software. The Ganzfeld dome, the pattern screen and generator, the amplifier and the Software used were part of the *BM6000-MAXI Electrophysiology Unit* (Biomedica Mangoni, Pisa, Italy). The stimuli were always carried on (two replicate recordings for each stimuli) in the same order: PERG right and left eye, fVEP right and left eye and ffERG right and left eye (Light-adapted 3.0 ERG, Dark-adapted 0.01 ERG with 20 minutes of adaptation, Dark-adapted 3.0 ERG with no further adaptation). Both eyes were investigated separately and the contralateral eye was covered to prevent light exposure. Each experimental session, including the animals' preparation, lasted between 3 and 3.5 hours; at the end of the electrophysiology sessions, the animals were gently recovered form anesthesia. The electrodes, the sutures and the blepharostat were removed and Chloramphenicol 10 mg-Sodium colistimethate 180,000 U.I.-Tetracycline 5 mg ophthalmic ointment (Colbiocin; SiFi s.p.a. Catania, Italy) was applied on the conjunctiva. The animals were then moved to a dark and quiet room and the oro-tracheal tube was removed when the swallowing reflex reappeared. Animals completely recovered from anesthesia within a maximum of 2 h, and were then moved back to their origin pen.

2.3.3 Statistical analysis

Data, acquired as single waves, were analyzed by OriginPro 9.1. A 50 Hz low-pass FFT filter was used for the ffERGs and fVEP waves, while pattern ERG recordings were filtered with a 5-20 Hz band-pass FFT filter. For ffERG, the amplitude (µV), from a to b waves was measured; for PERG we measured the N35-P50 peaks amplitude; for fVEP we measured N2-P2 peaks amplitude. Data were analyzed with R and with MedCalc Statistical Software version 15.11.0 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2015). All data were tested for normality by the Shapiro-Wilk test. Non-parametric tests (Wilcoxon test for paired samples and Mann-Whitney *U*-test for independent samples) were used for all electrophysiological variables.

2.4 Necropsy

Three animals treated with IAA and three controls untreated animals were examined at necropsy promptly after 30 min from euthanasia to avoid autolytic artifacts. The whole ocular globes and brain were extracted for the sampling.

2.5 Histological evaluation of the retina

The whole ocular globes were fixed in 10% formalin and routinely trimmed with a sagittal section performed perpendicular to the long posterior ciliary artery an adjacent to the optic nerve, including the superior and inferior retina (Wilcock and Njaa, 2016). Sections 4 µm thick were cut from paraffin-embedded tissue and routinely stained with hematoxylin and eosin (HE).

2.5.1 Image analysis of the retina

Photographs were taken with a Leica microscope coupled with a camera device DFC 320 at 40x magnification (Leica Microsystems GmbH, Wetzlar, Germany).

Photographs of the retina were acquired in 6 topographical points:

- Visual streak, dorsal central: dorsal, 2 mm from the optic nerve head (ONH).
- Dorsal periphery: dorsal, 2 mm from the ora serrata.
- Dorsal mid-periphery: dorsal, mid point between ONH and ora serrata.
- Ventral central: ventral, 2 mmfrom the ONH.
- Ventral periphery: ventral, 2 mm from from the ora serrata.
- Ventral mid-periphery: ventral, mid-point between ONH and ora serrata.

A topographical map of the measured points is reported in Fig. S2.

For each points were measured the retina in the whole thickness from the photoreceptors layer to the inner limiting membrane (whole retina), the outer segment of the retina, from the photoreceptors layer to the outer plexiform layer (outer retina) and the inner segment of the retina, from the inner nuclear layer to the inner limiting membrane (inner retina). Furthermore each individual layer was measured at the different topographical points. The retinal pigment epithelium was excluded from the measurement because of the artifactual detachment of the retina. The thickness values were obtained from the measurements of photographs at high power fields (400x), using IC Measure 2.0.0.161. The retinal ganglion cells (RGC) density was evaluated by considering the number of retinal ganglion cells per retinal length (200 micron) in the 6 anatomical points/per eye (central, periphery and mid-periphery both dorsal and ventral). Data were not normally

distributed and were analyzed with non-parametric tests (Mann Whitney *U*-test and Kruskal Wallis test).

2.6 Histological evaluation of the optic pathways

After opening of the skull, the brain tissues were gently removed; the cerebral ventricles were opened, and tissues exposed to favor the subsequent fixation (10% formalin). Samples of the left/right optic tracts and the left/right lateral geniculate bodies were routinely processed, embedded in paraffin, cut into 4-µm thick sections and stained with HE. Only for geniculate bodies and adjacent diencephalon, half of the sample was fixed in 4% paraformaldehyde for at least 72 h then post fixed in the same fixative for 4 h. After rinsing in phosphate buffer saline (PBS, pH 7.4), the tissue was cryoprotected in 30% sucrose solution in PBS (pH 7.4) at 4 °C and cut on a sliding freezing microtome in serial coronal sections (50 µm). Sections were stored in PBS (pH 7.4) containing sodium azide (0.01%) until thionin staining.

Sections were analyzed using a Leica DMRB microscope. Brightfield images were acquired by means of a Polaroid DMCdigital camera (Polaroid Corporation, Cambridge, MA, USA) and DMC 2 software. Contrast and brightness were adjusted to reflect the appearance of the labeling seen through the microscope using Adobe Photoshop CS3 Extended 10.0 software (AdobeSystems, San Jose, CA). To calculate the neuronal density thionin-stained somata were plotted in every fifth section throughout each lateral geniculate nucleus with a computer-aided digitizing system (Accustage 5.1, St. Shoreview, MN). The boundaries of the lateral geniculate nucleus was drawn from the thionin-stained sections using a stereo microscope equipped with drawing tube. The outlines were superimposed on computer generating plots using Corel Draw X3 (Corel corporation, Ottawa, Ontario, Canada). The area measurements were done from the line drawings by using AxioVision Rel.4.8 (Zeiss). The neuronal density in the lateral geniculate nucleus was calculated as number of somata/mm2 in each section separately. For each nucleus, 5 sections were analyzed. The neuronal counts are expressed as the mean number of somata/mm2 ± standard deviation (SD), and Student's t-test was used for comparing data from control and IAA treated animals, with a significance level of $p < 0.05$.

2.6.1 Immunohistochemistry

Three serial sections, from each eye, optic tracts and the lateral geniculate bodies of 3 treated (IAA) and 3 control (C) animals , underwent immunohistochemistry (IHC) using antibodies to Glial Fibrillary Acidic Protein (GFAP) (Rabbit polyclonal antibody, catalog number Z0334, Dako,

Glostrup,Denmark, dilution 1:8000), Oligodendrocyte Lineage Transcription Factor 2 (OLIG2) (Rabbit polyclonal antibody, catalog number AB9610, Merck-Millipore, Billerica, MA,USA, dilution 1:500), Ionized calcium-binding adapter molecule (Iba1) (Goat polyclonal antibody, Novus Biologicals, Abingdon, OX14 3NB, UK, catalog number NB100-1028, dilution 1:2000) and Ki67 (Mouse monoclonal antibody, clone MIB1, DAKO, dilution 1:600). Sections were dewaxed and rehydrated. Endogenous peroxidase was blocked by immersion in 3% H_2O_2 in methanol for 30 min at room temperature. For detection of GFAP, enzymatic antigen retrieval was performed with 0.05% trypsin for 20 min at 37 °C. Thermic antigen retrieval for detection of Iba1 (citrate buffer pH 6.0) and OLIG2 (EDTA buffer pH 8.0) was performed by incubation for 10 min in microwave at 750 W.

All antibodies were incubated with the tissue sections overnight at 4 °C. Immunoreactivity was revealed by using secondary biotinylated antibodies (dilution 1:200) and amplified using a commercial avidin-biotin-peroxidase kit (VECTASTAIN® ABC Kits, Peterborough, UK). The chromogens 3,30-diaminobenzidine (0.05%) (DAB Chromogen/Substrate Kit K001, DS-4011-A, Diagnostic BioSystems, Pleasanton, CA, USA) and AEC (Dako, AEC Substrate Chromogen, Glostrup, Denmark) were used. Slides were counterstained with Papanicolaou's and Mayer's hematoxylin. The primary antibody was replaced with an irrelevant, isotype-matched antibody as a negative control. A porcine lymph node with granulomatous lymphadenitis was used as a positive control for Iba1. Two sections of normal porcine brain were used as positive control for GFAP and OLIG2.

2.7 Quantitative analysis of immunohistochemical stain

Sections were obtained from left and right optical tracts and left and right lateral geniculate nuclei of controls and IAA treated animals. For each section 5 photographs at high power field (400x) were acquired using a Nikon Digital Sight SD-MS camera (Nikon Corporation, Japan) connected to an optical microscope. Photomicrographs of 5 fields per each histological section of left/right optic tract and left/right lateral geniculate body of IAA-treated and control animals were captured at 40x magnification using a Nikon Digital Sight SD-MS camera (Nikon Corporation, Japan) connected to an optical microscope. The cell counter function included within the ImageJ 1.46 software was employed to count the three types of glial cells (astrocytes, oligodendrocytes and microglial cells). Cells were counted per 0.237 mm^2. Data of GFAP quantitation were normally distributed, and parametric test (Student's *t*-test for unpaired samples) was used for comparison between controls and IAA-treated animals. Data of IBA1 and OLIG2 were not normally distributed and non parametric test (Mann Whitney *U*-test) was used for comparison between controls and IAA-treated animals. Analyses were performed by CSS software (Statsoft, TULSA, USA) statistics.

Data concerning the percentage of the image covered by GFAP immunoreactivity were calculated according Bianco et al. (2015) and obtained using the automatic threshold algorithm of ImageJ (version IJ 1.46r downloaded from http://imagej.nih.gov/ij/download.html). For this analysis images were taken using a Leica DMRB microscope using identical acquisition parameters. For each animal, 5 photographs per each tract (left and right optic tract, left and right lateral geniculate body) were analyzed.

3 Results

3.1 Recovery from anesthesia and IAA treatment

The animals recovered from the treatment without observable side effects, the hematologic and biochemical analyses did not show any statistical differences ascribable to the IAA treatment (12 mg/kg). The alteration in time of some variables (RDW, MCH, MCV) is the result of the physiological growth of the animals. The data are reported in Supplementary Tables S1 and S2.

3.2 Electrophysiological analysis

Fig. 1A-E shows waveform responses to light stimuli (1A-E) before (T0) and one month (T30) after IAA treatment (n=11). Amplitudes, expressed in µV, of full-field electroretinogram (ffERG), pattern electroretinogram (PERG) and flash visually evoked potentials (fVEP) recordings are reported in Table 2, while latencies, expressed in ms, are shown in Supplementary Table S3.

After IAA treatment, all responses to light stimuli were significantly impaired. The rod response (dark adapted 0.01 ERG) was completely abolished, while the cone response (light adapted 3.0 ERG) was minimally preserved (1F) with an average value of 13.26±9.8 µV. The visual cortical response (fVEP) was also minimally preserved (average 3.9±3.7 µV), while the retinal ganglion cell (RGC) response (PERG) was abolished. No differences were observed between the right and the left eyes. Peak latencies for all variables were consistent among animals at T0 and T30, but differed after treatment due to the peaks amplitudes reaching the noise level.

3.3 Histological evaluation of the retina

The retina was normally stratified in the control animals (Fig. 2A). Bilaterally, 2 mm dorsal to the optic disc in the area of visual streak, the iodoacetic acid-treated pigs showed a selective atrophy of rods with sparing of cones and decreased cellularity of outer nuclear layer (early retinal atrophy) (Fig. 2B). Ventrally, in the central, mid-point and peripheral retina, and dorsally at the mid-point and peripheral retina, consistently across the analyzed animals, there was a complete loss of the outer plexiform layer, outer nuclear layer and photoreceptor layer (end stage retinal atrophy) (Fig. 2C). The retinal pigmented epithelium, the inner nuclear layer, the inner plexiform layer, the ganglion

cells and nerve fibers layers were not affected. The whole retinal thickness was decreased for the treated animals compared to the controls in both eyes (right eye p=0.019, left eye p=0.022, Mann Whitney *U*-test). The atrophy was selective to the outer retina rather than the inner retina in the treated animals in both eyes compared to the controls (outer retina right eye p<0.0001 , left eye p<0.0001; inner retina right eye p=0.932 left eye p=0.761, Mann Whitney *U*-test). The measurement of the whole, outer and inner retina, in the central, peripheral and mid-point retina dorsally and ventrally in each treated and untreated animal is reported in Table 3 and Fig. S3.

In order to assess the intra-individual variability the analysis of the measurements in the different retinal locations (central, mid-point and peripheral both at the dorsal and ventral level) in each subject was assessed and no statistically significant difference was found in the different points (Right p=0.172 Left 0,497 Kruskal Wallis test).

No relevant pathological changes were detected in other than retinal ocular anatomic structures. The count of retinal ganglion cells/per retinal lenghtdid not reveal any differences between the normal and IAA treated retinae in both eyes (right p= 0.34, left p=0.17 Kruskal Wallis test)) (Supplementary Table S4). Futhermore, no intraindividual variability was detected in the count of RGCs at the different retinal points (central, mid-point and peripheral both at the dorsal and ventral level) (Right p= 0.052 Left p= 0.116 Kruskal Wallis test). Immunohistochemistry with antibody to Iba1 highlighted rare and scattered round microglial cells without ramification (ameboid microglia) in the retina of control animals (Fig. 3A). Numerous microglial cells with prominent ramifications (activated microglia) were observed in areas of selective atrophy of rods (early retinal atrophy) (Fig. 3B1) compared to the normal retinae. The microglial cells in the affected retinae were more numerous in the optic disc, and occasionally formed multifocal aggregates (glial nodules). Absence of microglial cells was observed in areas of end stage retinal atrophy (Fig. 3B2). GFAP revealed regular aligned Müller cells across the normal retina (Fig. 3C). The Müller cells in the atrophied retinae (Fig. 3D) were activated and haphazardly arranged, with loss of the regular columnar alignment and effacement of the retinal layers (glial scar). No immunoreactive for Ki67 has been detected in all tested eyes (not shown). OLIG2 did not detect any positive oligodendrocytes both in the controls and in the atrophied retinae (Fig. 3E and 3F).

3.4 Histologic evaluation of the optic pathways

Regularly structured optic tract was present in the control animals (Fig. 4A). A mild satellitosis of the optic tract along with a mild to moderate gliosis of the optic nerve were detected. The histological sections of the optic tracts of IAA treated pigs were characterized by dyskaryosis of the

glial cells compared to the controls (Fig.4A and 4B). No differences about the number of microglial cells were detected in control and IAA treated animals in the optic tract (p= 0.84; Mann Whitney *U*-test). The astrocytes soma and endfeet were highlighted (Fig. 4E-4F) for the area evaluation. Comparing the area between the treated and controls animals, there was a minimal statistically significant difference (p=0.04 t-Student), which was tendentially lost if the right and left eyes were considered separately (right p=0.17, left p=0.10 t-Student).. Oligodendrocytes did not shown differences in control and IAA treated animals in the optic tract (p=0.76 Mann Whitney *U*-test).

The histological sections of the lateral geniculate bodies did not reveal pathological changes in control and IAA treated animals (Fig. 5A and 5B). Microglial cells were significantly decreased in IAA treated compare to the control (p= 0.02, Mann Whitney *U*-test) (Fig. 5C and 5D). The astrocytes soma and endfeet were highlighted (Fig. 5E-5F) for the area evaluation. Comparing the area between the treated and controls animals, there was a minimal statistically significant difference (p=0.03 t-Student), which was tendentially lost if the right and left eyes were considered matched (right p=0.02, left p=0.42 t-Student). (p= 0.10 Compared to the controls, a decrease number of oligodendrocytes in the IAA treated animals was counted (p= 0.0004, Mann Whitney *U*-test) (Fig. 5G and 5H). The median count of microglial cells and oligodendrocytes and the area covered by astrocytes was measured and expressed as percentage, in IAA and control groups are summarized in Table 4. In thionin preparations, the neuronal population of the lateral geniculate nucleus was made up in its grater part of polygonal neurons, but also included fusiform and spheroidal neurons. Although the lateral geniculate nucleus had a rather striated appearance, it was difficult to recognize the existence of a clear laminar organization. As reported in Supplementary Table S4, the neuronal density did not reveal any differences between control and IAA treated animals.

4 Discussion

We utilized a high dose 12 mg/kg IAA in 11 hybrid pigs to mimic an end-stage retinal degeneration. Animals were evaluated 30 days after treatment with a panel of functional tests (ffERGs, PERG and fVEP) and three were euthanized together with three control animals for the histological analysis. It is important to acknowledge that, while setting up the right protocol for retinal degeneration induction by means of IAA, three animals died immediately after the administration of the chemical. This was potentially due to the fact that the IAA solution was prepared and immediately stocked at -20 °C, and thawed and warmed up to 37 °C on the day of administration. Fresh preparation of the solution on the day of administration, as reported in the

Material and Methods section, allowed us for a safe procedure avoiding mortality. The hypothesis is therefore related to the stability of the IAA once solubilized.

From a functional point of view, the 12 mg/kg dose of IAA was effective in reproducing the end-stage retinal degeneration in swine. Light adapted and dark adapted (0.01 and 3.0) ERGs confirmed previously reported results, with cone responses partially preserved 30 days after treatment (Wang et al., 2011; Noel et al., 2012). On the contrary, rods responses evoked by dark-adapted 0.01 stimuli were completely abolished, confirming that rods are more sensitive than cones to the IAA-induced block of glycolysis (Wang et al., 2011). The vision impairment assessed by ff-ERG is coherent with our previous behavioral study, where pigs were challenged with the obstacle course test to assess vision 30 days after IAA treatment (Barone et al., 2018). The results of PERG analysis seem to be indicative for a complete impairment in functionality of RCGs, while the results of fVEP analysis still suggest residual functionality of the optic pathway.

The discussion regarding the PERG results is quite challenging as, to the best of the author's knowledge, few information is available regarding its application and interpretation in pigs. Pattern electroretinograms (PERG) were successfully recorded in animals; we were able to measure strong and stable signals with wave morphology and average amplitude (N35-P50) very close to human. Our results demonstrate that there is a high variability between animals, but that the wave morphology is very consistent. In 2001, Janknecht and collaborators performed PERG measurements in pigs, but the amplitudes were too small to be recognized in the original traces (Janknecht et al., 2001). Pattern ERG is a retinal biopotential evoked by a temporally modulated patterned stimulus of constant mean luminance, which largely arises in RGCs (Bach et al., 2013). The results are coherent with a previous study that described the PERG in normal pigs at various spatial frequencies to detect the spatial resolution of porcine retina (Barone et al., unpublished data). PERG reflects the integrity of bipolar cells and RGCs and therefore is an important tool for the evaluation of possible toxic effects of the IAA other than selective photoreceptor degeneration. We found that, 30 days after IAA treatment, PERG amplitude was decreased by 98.2%. This result is in contrast with the histological finding of a preservation of INL and GCL layers throughout the retina. However, it must be considered that PERG is clinically used to objectively evaluate visual acuity as a direct measure of ganglion cell function, and monitor alteration caused by conditions such as glaucoma, a condition where the photoreceptors are still preserved (Holder, 2001). No data are available for PERG recorded in pigs with photoreceptor degeneration. It is reasonable that, in the swine species, PERG reflects the condition of the central streak, a fovea-like area with a high

density of cones (Hendrickson and Hicks, 2002). Although the light adapted response (light adapted ERG 3.0) is partially preserved after the IAA lesion, we were not able to detect a PERG response 30 days after the treatment. We can assume that the residual response was too weak to be detected or that the IAA affects the functionality, but not the morphology, of RGCs. Further studies are therefore necessary to better understand this phenomenon.

In the present study, fVEP were recorded to investigate the functional integrity of the visual pathways using a subdermal needle as active electrode placed close to the occipital protuberance. Previous attempts to record VEP in laboratory pigs were performed using epidural recording electrodes with a neurosurgical approach (Schwahn et al., 2001; Laube et al., 2002; Sachs et al., 2005). However, this approach is invasive and requires specific skills and tools. Our approach, with the standard technique described in the ISCEV guidelines, is indeed more easy and much less invasive, as it does not require any surgical preparation (Odom et al., 2016). To overcome the anatomical issues of the pigs, which have a thick pneumatized skull bone and heavy neck muscles and fatty rind in adult age, we enrolled 32±12 kg body weight animals in the trial. We were able to record high amplitudes VEP from animals that didn't exceed 50 kg body weight providing a step forward in the refinement of the pig model in preclinical ophthalmology and neuroscience. Nonetheless, when it comes to the interpretation of the results of this analysis, especially 30 days after the induction of photoreceptor degeneration, the lack of background data represents a critical point. Indeed, the significative decrease in fVEP response does not reflect the results of the histological evaluations, where anatomy seems to be preserved. The weak fVEPs signal recorded 30 days after IAA administration could be the result of a combined photoreceptors/RGCs dysfunction in localized regions of the retina, since also PERGs were almost completely abolished. More advanced investigations should be performed in order to be able to build and support a strong hypothesis.

From a morphological point of view, the toxic effect of IAA on the porcine retina determined a selective rod loss as previously described (Wang et al., 2011). We observed a rod loss with sparing of cones and decreased cellularity of the outer nuclear layer (interpreted as early retinal atrophy) selectively at level of the central retina in the visual streak. Dorsal periphery and ventral central and periphery, the retina showed a complete loss of the outer and photoreceptors layers (interpreted as end stage atrophy). The differences between the two areas were ascribed to the peculiar anatomic structure of the porcine retina, which contains a cone-dominant central visual streak and is enriched of rods in the peripheral retina (Chandler et al., 1999; Hendrickson and Hicks, 2002;

Kostic and Arsenijevic, 2016). The selective targeting of rods by IAA, explains the more severe atrophy in the peripheral retina, where rods predominate. The mechanism of action of IAA on photoreceptor degeneration is demonstrated by a significant shortening of the outer retina in the treated animals where photoreceptor nuclei and their processes reside (Dubielzig et al., 2010). The response to the degeneration was represented by a clear activation of microglial cells (ameboid type), absent in the control retinas. Microglial cells have phagocytic functions in the central nervous system and retina and are required for neuronal homeostasis and innate immune defense (Langmann et al., 2007). Microglia activation is reported to be associated with human retinal dystrophies (Langmann et al., 2007; Nakatake et al., 2016). Based on our findings, microglial proliferation was replaced by macroglial cells, which gradually obscured the retina by forming a glial scar.. This feature can be explained by the peculiar energetic metabolism of macroglial cells, which are resistant to anoxia or absence of glucose in vitro (Winkler et al., 200). Müller cells proliferation, without a consistent Ki67 immunoreactivity, is reported in the rabbit treated with IAA (Liang et al., 2008) but, it has never been described in the swine models in the optic pathways. Conversely, the astrocytes covered area didn't show any signifanct differences in treated animals in the optic pathway compared to the controls. Further studies are needed to understand the pathogenetic mechanism of and the reactive changes induced by IAA. Oligodendrocytes were not present in the retina but were decreased in number in the lateral geniculate bodies of IAA-treated animals. Considering the reduced number of the analyzed animals and the lack of known cut off value in the literature regarding healthy pigs, this finding should be further investigated in order to exclude the presence of interindividual variability. Oligodendrocyte loss could be explained by a toxic effect, probably driven by the IAA-induced glycolysis inhibition (Wang et al., 2011). No data in the literature confirm this subcellular toxic effect and further experiments are needed to confirm this hypothesis. Inhibition of glycolysis and cell stress, confirmed by 1H-NMR vitreous humor characterization in pigs after 30 days of IAA treatment (Elmi et al., 2019), may provoke three types of events: apoptosis, necrosis or autophagy. Further investigations of the mechanisms of cell death could elucidate the toxic effect of IAA on photoreceptor degeneration.

5 Conclusions

Overall, the IAA treatment leads to selective rod and partial cone degeneration with preserved morphology of the inner retina and optic pathways. Nonetheless, from a functional point of view, RGCs and the optic pathways seem to be partially impaired as suggested by the significative

decrease in PERG and fVEP responses. Our study contributes to the understanding and better characterization of a rapid and cost-effective pig model of photoreceptor degeneration that might be very useful for future applications of retinal prostheses or end-stage disease treatment approaches.

Competing Interests: All authors declare that they have no competing interests.

Funding: This work was supported by UNIBO [RFO ex 60% to M.L.B.]; Telethon-Italy [GGP14022 to G.P. and F.Be.]; and the Italian Ministry of Health [RF-2013-02358313 to G.P.].

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Figure legend

Figure 1. Representative waveforms for ffERG, PERG and fVEP.

Light adapted 3.0 (1A) dark adapted 0.001 and 3.0 (1B,C), pattern ERG (1D) and flash VEP (1E) waveform before (T0) and 30 days after 12 mg/kg IAA treatment (T30). 1F shows normalized to baseline amplitude after treatment (Wilcoxon for paired samples, p value≤0.001) for all the variables. The amplitudes of the responses were calculated as Δab for ffERG, ΔN35P50 for PERG and ΔN2P2 for fVEP.

Figure 2. Hematoxylin-eosin staining of the retina.

Nerve Fiber Layer (NFL); Ganglion Cell Layer (GCL); Inner Plexiform Layer (IPL); Inner Nuclear Layer (INL); Outer Plexiform Layer (OPL); Outer Nuclear Layer (ONL); Photoreceptors layers (PL); Retinal Pigment Epithelium (RPE). A) Normal stratified porcine retina, 40x. B) Selective atrophy of rods in a pig treated with iodoacetic acid. The cones are spared and there is a decreased cellularity of the outer nuclear layer, 40x. C) End stage retinal atrophy. Complete loss of the outer plexiform layer, outer nuclear layer and photoreceptor layer. The inner retina is spared. Calibration bar = 100 µm.

Figure 3. Swine, retina, immunohistochemistry to Iba1, GFAP and OLIG2.

A. Few positive ameboid microglial cells (arrow) in the normal retina, DAB, 40x. B1. Numerous activated microglia (arrows) scattered throughout the injured retinal layers, DAB, 40x. B2. Endstage atrophy with microglial cells depletion, DAB, 40x. C, D. Swine, retina, immunohistochemistry to GFAP. Positive stained macroglia normally arranged across the normal retina, AEC, 40x. Atrophic retina composed of reactive macroglial cells, forming a glial scar, AEC, 40x. E, F. Swine, retina, immunohistochemistry to OLIG2. Absence of oligodendrocytes in the normal retina, AEC, 40x. Absence of oligodendrocytes in the atrophic retina, AEC, 40x. Calibration bar = 100 µm.

Figure 4. Pig optic tract HE, immunohistochemistry to Iba1, GFAP and OLIG2.

A, B: Pig, optic tract, control-case and IAA, hematoxylin and eosin (HE) staining. Dyskaryosis of glial cells (arrows) in the optic tract of IAA animal compared to the control, DAB, 20x.

C, D: Pig, optic tract, control-case and IAA, Iba1 immunostaining; no differences in the number of microglial cells in the IAA and control case, DAB, 20x.

E, F: Pig, optic tract, control-case and IAA, GFAP immunostaining; intense diffuse GFAP immunoexpression in soma and endfeet of astrocytes, DAB, 20x.G, H: Pig, optic tract, control-case

and IAA, OLIG2 immunostaining; no differences in the number of oligodendrocytes in the IAA and control case, DAB, 20x.

Calibration bar = 100 µm.

Figure 5. Lateral geniculate body, immunohistochemistry to Iba1, GFAP and OLIG2.

A, B: Pig. Lateral geniculate body, control-case and IAA, hematoxylin and eosin (HE) staining, 20x. No morphological changes in the IAA and control case, HE, 20x.

C, D: Lateral geniculate body, control-case and IAA, Iba1 immunostaining. A decreased number of microglial cells in the IAA compared to the control case, DAB, 20x.

E, F: Pig. Lateral geniculate body, control-case and IAA, GFAP immunostaining, DAB, 20x. Intense diffuse GFAP immunoexpression in soma and endfeet of astrocytes, DAB, 20x.

G, H: Pig. Lateral geniculate body, control-case and IAA, OLIG2 immunostaining. A significant decrease number of oligodendrocytes in the IAA compared to the control case, DAB, 20x.

Calibration bar = 100 µm.

Table 1. Stimuli and recording parameters for swine-adapted ISCEV Standard ffERGs, pERG and fVEP.

ffERG test	Adaptation/ background strength and time	Stimulus strength cd•s•m^{-2}	Inter stimulus time	Recording bandpass	Main physiological generator(s)
Light-adapted 3.0 ERG	30 cd m^{-2} = 10 min	3	1.1 Hz	2 - Hz - 500	*a-wave*: cones with post-receptoral on & off pathways *b-wave*: on & off bipolar cells
Dark-adapted 0.01 ERG	DA = 20min	0.01	0.49 Hz	1 - Hz - 500	*b-wave*: rod-initiated on pathways
Dark-adapted 3.0 ERG*		3	0.1 Hz	1 - Hz - 500	*a-wave*: combined response photoreceptors & post-receptoral on pathways *b-wave*: on & off bipolar cells

Analysis	Stimulus	Luminance	Contrast	Presentation rate	Recording bandpass
Pattern ERG	Vertical square 0.10 C/°	47 cd m^{-2}	96 %	1.98 rps (0.5 Hz)	2 - Hz - 200
Flash VEP	Flash	3 cd•s•m^{-2}	-	1 Hz	1 - Hz - 500

* Dark-adapted ERGs are recorded sequentially without further dark adaptation. Thus, only the 0.01 ERG is a fully dark-adapted response

Table 2. Amplitudes for ffERG, PERG and fVEP before and after IAA treatment.

Stimulus		T0		T30		p value
		n	Median (min-max)	n	Median (min-max)	
Light-adapted 3.0 ERG	right	11	264.0 (146.0-394.0)	11	12.0 (0.0-30.0)	0.001
	left	11	226.0 (125.0-326.0)	11	9.0 (0.0-28.0)	0.001
	total	11	263.0 (144.5-351.0)	11	11.2 (1.9-27.0)	0.001
Dark-adapted 0.01 ERG	right	11	81.0 (40.0-253.0)	11	0.0 (0.0-4.0)	0.001
	left	11	119.0 (40.0-137.0)	11	0.0 (0.0-4.0)	0.001
	total	11	96.6 (40.0-189.5)	11	1.5 (0.0-2.5)	0.001
Dark-adapted 3.0 ERG	right	11	311.0 (120.0-413.0)	11	18.0 (0.0-39.0)	0.001
	left	11	273.0 (182.0-413.0)	11	15.1 (0.0-59.0)	0.001
	total	11	325.5 (166.5-412.0)	11	16.5 (0.0-47.5)	0.001
PERG 0.1 cy/deg	right	10	10.5 (3.0-16.0)	10	0.0 (0.0-1.0)	<0.001
	left	10	6.0 (2.4-19.5)	10	0.0 (0.0-1.0)	0.002
	total	10	9.3 (3.5-17.8)	10	0.2 (0.0-0.5)	0.002
Flash-VEP	right	11	25.0 (8.5-52.4)	11	2.0 (0.0-12.3)	0.001
	left	11	15.5 (3.7-57.3)	11	3.4 (0.0-12.3)	0.001
	total	11	20.3 (6.5-53.3)	11	2.6 (0.0-7.9)	0.001

Cones (light adapted 3.0 ERG), rods and combined (dark-adapted 0.01 and 3.0 ERG), RGCs (PERG) and visual cortical (fVEP) response to light stimuli: a-b amplitudes for ffERG, N35-P50 amplitude for PERG and N2-P2 amplitude for fVEP are reported. Total referes to the averaged date of both eyes. Data are expressed in µV, median, minimum and maximum are given and a non-parametric test (Wilcoxon for paired samples) was used to calculate P values.

Table 3. Retinal thickness in control animals and iodoacetic acid treated animals.

Retinal Thickness (µm)	CTR		IAA		p value	
	Right	Left	Right	Left	Right	Left
Whole Retina	204.17 (99.30-347.60)	202.94 (117.16-349.72)	155.28 (68.34-270.38)	153.29 (68.86-416.20)	0.019	0.022
Outer retina	86.12 (50.30-129.94)	85.90 (51.56-191.18)	27.55 (11.04-102.98)	30.02 (14.22-61.44)	<0.0001	<0.0001
Inner Retina	106.63 (48.38-273.04)	109.57 (57.52-160.20)	112.26 (56.02-167.40)	122.80 (54.64-354.76)	0.932	0.761

Measurements of the retinal thickness in the whole, outer and inner retina in the left and right eyes. Data are reported as median (minimum-maximum); Mann Whitney *U*-test was used to calculate the p values. CTR: control group (n=3); IAA: iodoacetic acid group (n=3).

Table 4. Count of microglial cells and oligodendrocytes and area of astrocytes in the optic tract and lateral geniculate nucleus of control animals and iodoacetic acid treated animals.

Glial cells		CTR		IAA		p value
		n	Median (min-max)	n	Median (min-max)	
OT	Iba1	3	8.7 (8.5-11.7)	3	9.5 (7.4-15)	0.84°
	GFAP	3	32.9 (18.6-42.5)	3	28.7 (19.5-44.5)	0.04*
	OLIG2	3	13.5 (11.8-18.7)	3	14.4 (12.5-22.2)	0.76°
LGN	Iba1	3	12.6 (8.9-13.8)	3	9.7 (8.8-9.8)	0.028°
	GFAP	3	27.7(18.1-42.4)	3	26.2 (12.2-41.3)	0.03*
	OLIG2	3	17.3 (14-18.2)	3	11.6 (10.6-14)	0.00042°

OT: optic tract; LGN: lateral geniculare nuscleus. The glial cells (microglial cells, astrocytes and oligodendrocytes) were highlighted using immunohistochemistry to Iba1, GFAP, and OLIG2. Median, minimum and maximum are given and * Student's *t*-test; ° Mann Whitney *U*-test was used to calculate the p values. CTR: control group (n=3); IAA: iodoacetic acid group (n=3).