

**1H NMR Spectroscopy Characterization of Porcine Vitreous Humor in Physiological and Photoreceptor Degeneration Conditions**

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Purpose: Qualitative and quantitative analysis of vitreous humor (VH) is important to discriminate between physiological and pathological conditions and may be particularly helpful when using animal models for ophthalmologic research. The aim of the present study was to investigate the physiological qualitative/quantitative composition of the VH of the standard pig using a metabolomics approach based on 1H nuclear magnetic resonance (NMR) spectroscopy. A secondary aim was the characterization of the VH of the porcine model of photoreceptor degeneration induced with iodoacetic acid (IAA), widely used in the biomedical field, and comparison with the physiological one.

Methods: VH samples were collected from 30 pigs (17 in the physiological condition and 13 treated with IAA) upon vitrectomy and analyzed by means of 1H NMR spectroscopy for characterization.

Results: On all analyzed samples, 40 molecules could be identified and quantified, with lactate being the most abundant in both physiological and photoreceptor degeneration conditions. Upon comparison, only 17 molecules showed statistical differences: In the IAA group, glucose and glutamine increased, while lactate, 4-amynobutyrate, hypoxanthine, dicarboxylic acids (succinate and fumarate), amino acids with their derivatives (creatine, alanine, valine, lysine, leucine, glycine, taurine, and isoleucine), and choline with its precursor sn-glycero-3-phosphocholine decreased.

Conclusions: The results validate the metabolic impairment determined by glycolysis inhibition upon systemic IAA administration. In conclusion, this work represents the first characterization of the porcine VH metabolome in physiological conditions and provides additional information for the characterization and refinement of the IAA-induced photoreceptor degeneration model.

Keywords: vitreous humor, photoreceptor degeneration, porcine model, 1H NMR spectroscopy, metabolomics

The vitreous humor (VH) is a highly hydrated (>98%) jellified extracellular matrix mainly constituted by different types of collagen fibrils, in particular II, V/XI, IX, and hyaluronan, a nonsulphate glycosaminoglycan (GAG) structure that fills the spaces between collagen fibrils. The VH is considered to be virtually acellular except for the presence of a small number of hyalocytes. When looking at its anatomic features, VH is enclosed in and attached to the pars plana of the retina and makes contact with the lens. VH has different functions including mediating the metabolic requirements of the lens, stopping the leakage of cells and large macromolecules from the vascular stream into the vitreous cavity, and acting as a diffusional barrier to the free movement of molecules between the anterior and posterior eye. Furthermore, VH seems to act as a reservoir for several compounds unable to cross the blood-retinal barrier (BRB) such as proteins and other solutes.

In recent decades, different studies have underlined the importance of the analysis of VH in order to find differences in its molecular composition during diverse physiological/pathological conditions such as diabetes, aging, and retinal diseases by means of different analytical approaches. Undeniably, qualitative and quantitative results are highly influenced by a correct sampling technique, which is actually obtained by vitrectomy during surgical procedures. Metabolomics based on 1H nuclear magnetic resonance (NMR) spectroscopy has proven to be one of the most favored and reliable approaches for in-depth qualitative and quantitative characterization of a variety of biological fluids in different species, including porcine species. For example, a 1H NMR-based topographical analysis of goat VH showed the existence of different
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Methods

Animals and Protocols

The use of the animals in this study was regulated by two protocols approved by the Italian Ministry of Health, one with Legislative Decree 116/92 and the other with the new Legislative Decree 26/2014, in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The procedures described below are part of a preclinical trial of a larger project aimed at developing new therapeutic approaches to restore vision in patients affected by pathologies that lead to photoreceptor degeneration.

Thirty commercial hybrid pigs ([Large White × Landrace] × Duroc) of both sexes, with weight between 16.6 and 38.2 kg (8–14 weeks old), were enrolled in this study. Thirteen (n = 13) pigs were treated, under general anesthesia, with 12 mg/kg IAA (CAS No. 64-69-7; Sigma Chemical Corp., St. Louis, MO, USA) as previously described by Wang et al.,28 to induce retinal degeneration. The remaining 17 untreated pigs (n = 17) were enrolled in the control group (CTR).

All animals were housed in multiple boxes with a 12:12-hour light:dark cycle and a minimum of 40 lux during the light period.26 Chains and wood were used as environmental enrichment.30 The pigs were fed with standard commercial diet (PROGE, Reggio Emilia, Italy) twice a day, with ad libitum water.

Sampling Procedure

Four weeks after IAA treatment, the VHpIAA were sampled during the surgical procedures, which were aimed to assess a novel therapeutic approach to restore vision. Before anesthesia, animals were fasted for 12 hours. After sedation with an intramuscular injection of tiletamine-zolazepam (Zoleti, 15 mg/kg; Virbac, Prague, Czech Republic), general anesthesia was induced with isoflurane (IsoFlo; Zoetis Italia s.r.l., Rome, Italy) administered through a mask in a 1:1 oxygen/air mixture, and maintained, after orotracheal intubation, with the same halogenated agent. From each animal, approximately 500 µL VH was collected upon vitrectomy into a sterile cryogenic tube and immediately frozen in liquid nitrogen, then stored at −80°C until analysis. The same procedures were performed on the CTR group animals.

Metabolomics by 1H NMR

Humor samples were prepared for NMR analysis by means of centrifugation for 15 minutes at 18,650g and 4°C. Secondly, 350 µL supernatant with 350 µL bidistilled water was added to 100 µL of a D2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mM, used as NMR chemical-shift reference, buffered at pH 7.00 by means of 1 M phosphate buffer. Finally, each sample was centrifuged again under the above conditions and the supernatant underwent analysis.

1H NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.15 MHz. Following Ventura and colleagues,12 the signals from broad resonances originating from large molecules were suppressed by a Carr-Purcell-Meiboom-Gill (CPMG) filter composed by 400 echoes with a τ of 400 µs and a 180° pulse of 24 µs, for a total filter of 330 ms. The deuterated water (HOD) residual signal was suppressed by means of presaturation. This was done by employing the cmgrp1d sequence, part of the standard pulse sequence library. Each spectrum was acquired by summing up 256 transients using 32 K data points over a 7184-Hz spectral window, with an acquisition time of 2.28 seconds. In order to apply NMR as a quantitative technique, the recycle delay was set to 5 seconds, keeping in consideration the relaxation time of the protons under investigation.1H NMR spectra were baseline-adjusted by means of the peak detection according to the “rolling ball” principle31 implemented in the baseline R package.32 A linear correction was then applied to each spectrum, so as to make the points pertaining to the baseline randomly spread around zero. Differences in water and fiber content among samples were taken into consideration by probabilistic quotient normalization,33 applied to the entire spectra array.

The signals were assigned by comparing simultaneously their chemical shift, multiplicity, fine structure, and signal relative intensity with the Human Metabolome Database34 and Chenomx software library (ver 8.1; Chenomx, Inc., Edmondston, AB, Canada), both built on pure compounds under the experimental conditions of the present work, as detailed in Figures S1 through S13. For molecules giving rise to a singlet only, such as formate, fumarate, glycine, pyruvate, or...
succinate, the assignment was accepted if the signal registered on the pure compound and the one under investigation were less than 0.6 Hz apart, as detailed in Figure S14. Integration of the signals was performed for each molecule by means of rectangular integration.35

Statistical Analysis

Data investigation was conducted on R platform.36 In metabolomics evaluation, molecules whose concentration varied in relation to treatments were tested by a nonparametric Mann-Whitney test, by applying a continuity correction in the normal approximation for the P value. For this purpose, a significance limit P of 0.01 (confidence interval 99%) was accepted. To highlight the underlying trends characterizing the samples, the principal component analysis model in its robust version (rPCA) was built on the molecule concentrations, centered, and scaled to unity variance, according to Damisch.37 This was done by taking advantage of the PCAHubert function of the R package rrcov.38 For each rPCA model, we calculated the scoreplot, the projection of the samples in the principal component (PC) space, tailored to highlight the underlying structure of the data. Additionally, the correlation plot was calculated, relating the concentration of each variable to the components of the rPCA model, therefore tailored to highlight the most important molecules in determining the trends highlighted by the scoreplot.

RESULTS

VH samples collected during vitrectomy proved to be suitable for the chosen analytic technique, as already reported in the literature.15

1H NMR spectra of VH of the CTR and IAA groups are reported in Figure 1. Forty molecules were identified and quantified in all of the analyzed samples, and the results including P values and trends are summarized the Table.

From a quantitative point of view, both in the CTR and IAA groups, lactate was the most concentrated metabolite, followed by glucose, glutamine, and ascorbate (Table). Other metabolites identified were the amino acids, with glutamine being the most representative. The comparison between the two experimental groups highlighted 17 significantly different molecules (P < 0.01). In order to obtain an overview of underlying trends, the above-mentioned 17 molecules were employed as basis for an rPCA model, shown in Figure 2. Along PC 1 of its scoreplot (Fig. 2A), representing as much as 87.2% of the entire sample variability explained by the PCA, the two groups appeared as significantly separated (P < 0.01).

The molecules that mainly drove such separation, with a correlation between importance over PC 1 and concentration higher than 0.8 (Fig. 2B), were glycine, taurine, choline, creatine, sn-glycero-3-phosphocholine, 4-aminobutyrate, hypoxanthine, and fumarate, more concentrated in CTR group.

DISCUSSION

In recent years, the use of pigs for non- or preclinical ophthalmologic trials has been exponentially increasing as proven by the literature.39 One of the biggest issues of enrolling animal models of any kind in such studies is the lack of extensive characterization that often affects the translational potential.40 This is why extensive characterization is key to interpret data and thus to obtain robust results, and to comply with the 3R principles for animal experimentation.41 Moreover, the possibility to highlight biomarkers of pathology in biological fluids of animal models may assist correlation with the actual human pathology, validating the model itself.

To the best of the authors’ knowledge, this is the first metabolomics characterization of the porcine VH by means of 1H NMR spectroscopy, a platform characterized by lower sensitivity compared to others usually employed for metabolomics investigations, counterbalanced by a high reproducibility and inherent simplicity.42 Such a physiological profile...
was used as reference for comparison with the VH metabolome of the swine model of photoreceptor degeneration induced by IAA. This model is indeed still lacking in-depth characterization, and the description of its VH metabolome may provide important information for future ophthalmologic experimental trials.

It is worth remarking on the importance of acknowledging the precise VH collection site since the different vitreous areas specifically reflect different metabolic activities influenced by the surrounding structures. The samples analyzed in the present study were collected upon vitrectomy and represent the portion of VH directly in contact with the retina, called the cortical area, providing direct insight into the retinal metabolic activity. The reproducibility of the sampling/handling technique was confirmed by the coherent identification and quantification, in all the samples, of the same 40 molecules as reported in the Table.

The physiological composition of the swine VH shows resemblance with what has already been reported in the literature for humans and animals with lactate being the most abundant metabolite. This is supposedly related to the limited availability of oxygen within the eye and high consumption rates in the retina that lead to anaerobic glycolysis with subsequent production of lactate. The other most abundant metabolites were glucose, glutamine, and ascorbate, as already reported, for example, in the goat. Glutamine seems to characterize the same vitreous area, the cortical area, also in the goat. This molecule represents the main metabolite of glutamate, which is the most important excitatory neurotransmitter in the retina and needs to be converted by glutamine synthetase in order to avoid neurotoxicity. Ascorbate seems to accumulate in the ocular tissues in order to prevent oxidative damage and its concentration is from 1 to 10 times higher than in the blood/plasma. It also seems to be involved in sunlight protection, as it is more abundant in diurnal rather than nocturnal animals.

The comparison between CTR and IAA groups has highlighted 17 molecules showing statistical differences, out of which only glucose and glutamine increased upon induction of photoreceptor degeneration. It is important to stress that systemic administration of IAA leads to the inhibition of 3-Methyl-2-oxovalerate 3.77
2-Oxoisocaproate 6.46
Succinate 1.35
Glycine 1.60
Acetate 2.84
Alanine 2.72
Valine 2.14
Lysine 2.00
Leucine 1.82
Formate 1.81
Tyrosine 1.78
Glycine 1.60
Pyruvate 1.44
Phenylalanine 1.36
Succinate 1.35
Lysine 1.20
Leucine 1.82
Formate 1.81
Tyrosine 1.78
Glycine 1.60
Pyruvate 1.44
Phenylalanine 1.36
Succinate 1.35
Isoeucine 1.15
Hypoxanthine 2.53
4-Aminobutyrate 0.15
Ethanol 0.70
Creatinine 0.70
Betaine 0.67
2-Oxoisocaproate 0.66
Choline 0.80
4-Hydroxyisobutyrate 0.39
3-Methyl-2-oxovalerate 0.37
Hexanol 0.37
sn-Glycerol-3-phosphocholine 0.32
Uracil 0.25
Dimethyl sulfoxide 0.19
Propionate 0.20
3-Hydroxyisovalerate 0.18
Fumarate 0.06
Isobutyrate 0.07
2-Hydroxyisobutyrate 0.05
Propylene glycol 0.03
anaerobic glycolysis,\textsuperscript{27} with subsequent degeneration of the photoreceptor as one of the most sensitive cells.\textsuperscript{21} Therefore, the explanation for the increase in glucose concentrations upon IAA administration seems to be clear, as a direct result of the relative inability to break it down for energy production. The other metabolite that showed a significant increasing trend was glutamine, the product of glutamate metabolism, which, in the retina, takes place in the Müller cells thanks to the action of the glutamine synthetase enzyme.\textsuperscript{47} When exposed to hypoxic damage, the retina shows an increase in glutamine concentrations due to the impossibility of the photoreceptors to correctly uptake glutamine from the extracellular matrix.\textsuperscript{48} Most importantly, Scott and colleagues\textsuperscript{21} demonstrated how systemic administration of IAA leads to proliferation of the Müller cells; therefore it is likely that higher levels of glutamine reflect higher metabolic activity of these cells and their enzymes.

The 15 molecules that significantly decreased in the IAA group included lactate, 4-aminobutyrate, hypoxanthine, dicarboxylic acids (succinate and fumarate), amino acids and their derivatives (creatine, alanine, valine, lysine, leucine, glycine, taurine, and isoleucine), and choline and its precursor sn-glycero-3-phosphocholine. As for what was already discussed regarding glucose, the decrease in lactate levels seems the obvious consequence of the inhibition of anaerobic glycolysis induced by the administered chemical compound. Indeed, as already stated, lactate is the main product of this metabolic pathway.\textsuperscript{49} 4-Aminobutyrate, also known as GABA, is a neurotransmitter produced in retinal horizontal cells using glutamate as substrate.\textsuperscript{50} Lower concentrations of GABA in the IAA group may be related to the low bioavailability of some precursor amino acids of the neurotransmitters, such as leucine, isoleucine, and taurine, that are indeed lower in this group. The same hypothesis can be proposed for the highlighted lower levels of another neurotransmitter precursor such as choline. When discussing the findings regarding GABA, it is also important to acknowledge the high levels of glutamine, which is a precursor in GABA production.\textsuperscript{47} It is indeed possible to hypothesize a potential inhibition to intracellular transport of glutamine leading to higher levels of precursor and lower levels of neurotransmitter. Further validation seems to be found in the levels of succinate, significantly lower in the IAA group, since it is a metabolite of GABA.\textsuperscript{50} Alongside fumarate, succinate also represents a reaction intermediate in the Krebs cycle, that provides the outer retina with enough ATP for its metabolic requirements and phototransduction.\textsuperscript{51} A decrease in these molecules may therefore strengthen the idea of an overall damage to the retinal structures and their metabolic pathways. Discussion regarding the overall decrease in amino acid levels can be complicated as they are involved in a variety of proteome-related processes.\textsuperscript{12} Nonetheless, this finding is a further reflection of the photoreceptor damage induced by IAA, as one of the roles of this molecule includes photoreceptor protection and development.\textsuperscript{52} Moreover, it has already been described how physiological VH is usually rich in amino acids as shed byproducts of retinal cell turnover.\textsuperscript{53} It is therefore likely that the induced decrease in photoreceptor metabolic activity may have led to reduced levels of free amino acids. The same hypothesis can be made for the reduction in hypoxanthine levels, related to an overall reduced retinal metabolism, as reported in a rat model of ischemic retinopathy.\textsuperscript{54}
In conclusion, this work represents the first characterization of the VH metabolome in young physiological standard pigs and in the IAA-induced model of photoreceptor degeneration through 1H NMR spectroscopy. The physiological characterization itself will provide a standard reference for future ophthalmologic non- and preclinical trials, potentially helping data interpretation and analysis. On the other hand, the findings regarding the IAA group represent a pivotal refinement of this important model and may contribute to more aware and responsible enrollment of such porcine models in future studies. Indeed, as for every animal model, in-depth overall characterization is necessary in order to achieve high translational value and robust and repeatable data.

Acknowledgments

Supported by RFO UNIBO, Telethon-Italy (Grant GGP14022), and the Italian Ministry of Health (Project RF-2013-02358313). Chenglin Zhu was supported by the Chinese Scholarship Council (Grant 201600901007).

Disclosure: A. Elmi, None; D. Ventrella, None; L. Laghi, None; G. Carnevali, None; C. Zhu, None; G. Pertile, None; F. Barone, None; F. Benfenati, None; M. L. Bacci, None

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