Behavioral Assessment of Vision in Pigs

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Swine (*Sus scrofa*) are often the 'gold standard' laboratory animal for ophthalmology research due to the anatomic and physiologic similarities between the porcine and human eye and retina. Despite the importance of this model, few tools for behavioral vision assessment in pigs are available. The aim of this study was to identify and validate a feasible and reproducible behavioral test to assess vision in a pig model of photoreceptor degeneration. In addition, a robust behavioral test will reduce stress and enhance enrichment by allowing animals opportunities for environmental exploration and by reducing the number of invasive experimental procedures. Two distinct behavioral approaches were tested: the obstacle-course test and temperament test. In the obstacle-course test, pigs were challenged (after an initial training period) to navigate a 10-object obstacle course; time and the number of collisions with the objects were recorded. In the temperament test, the time needed for pigs to complete 3 different tasks (human-approach, novel-object, and open-door tests) was recorded. The obstacle-course test revealed significant differences in time and number of collisions between swine with vision impairment and control animals, and the training period proved to be pivotal to avoid bias due to individual animal characteristics. In contrast, the temperament test was not altered by vision impairment but was validated to measure stress and behavioral alterations in laboratory pigs undergoing experimental procedures, thus achieving marked refinement of the study.

Abbreviations: ffERG, full-field flash electroretinography; HAT, human approach test; IAA, iodoacetic acid; NOT, novel object test; OC, obstacle course; ODT, open door test

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Swine (*Sus scrofa*) are often the 'gold standard' laboratory animal model for ophthalmology research due to the anatomic and physiologic similarities between the porcine and human eye and retina.²⁴ The swine retina contains a cone-dominant central visual streak comparable to the human fovea, with rods enriched in the peripheral retina.^{4,5} These characteristics make the pig retina an excellent model to study photoreceptor degeneration and retinal diseases.¹⁰ The first transgenic pig to model retinal dystrophy was generated by pronuclear microinjection to mimic a severe form of retinitis pigmentosa caused by the dominant rhodopsin (RHO) gene mutation p.P347L.²³ Since then, several transgenic models have been proposed, including the TgP23H pig generated through somatic cell nuclear transfer for the same human condition,²⁵ 2 models for Stargardt disease,²⁸ and one for cone-rod dystrophy (CORD6).¹¹ In addition to transgenic models, other proposed pig models for the ophthalmology field include the iodoacetic acid-induced model of photoreceptor degeneration, which is capable of mimicking mild to severe forms of retinitis pigmentosa.^{20,27,32}

Objective and subjective physiologic investigations are essential to assess vision in preclinical models, and it is imperative for researchers to use validated and feasible tools to measure vision and obtain robust and reproducible data. One of the best and most common ways to obtain reliable data when testing new therapeutic approaches in ophthalmology is objective evaluation by full-field flash electroretinography (ffERG). The

ffERG measures retinal responses under both light-adapted (photopic) and dark-adapted (scotopic) conditions and allows the investigator to evaluate responses between cones and rods differentially.^{14,33} Pigs are rapidly becoming the 'gold standard' preclinical model to test different therapeutic protocols such as gene therapy^{16,30} and artificial retinal prosthetics,^{1,13,26} which often require validation by ffERG analysis. However, when applied to pigs and other laboratory animals, ffERG requires anesthesia of subjects.^{2,19} A behavioral test, even if not entirely objective, could improve study design and be considered a refinement of the model by avoiding repeated anesthetic procedures.

The aim of the current study was to identify and validate a feasible and reproducible behavioral test to assess vision in a pig model of photoreceptor degeneration. In addition, a robust behavioral test will reduce stress and enhance enrichment by allowing animals opportunities for environmental exploration while reducing the number of invasive experimental procedures.

Materials and Methods

Animals. The use of pigs in this study was regulated by 2 protocols approved by the Italian Ministry of Health (D.Lgs 116/92 and D.Lgs 26/2014). The procedures described are part of a larger project aimed at developing new therapeutic approaches to restore vision in a pig model of photoreceptor degeneration.

Commercial hybrid pigs (Large White × Landrace × Duroc; $n = 22$; 12 female and 10 sterilized males; age [mean ± 1 SD], 9.8 ± 1.6 wk; weight, 31.6 ± 11.7 kg) were enrolled in the study. Of these, 17 pigs (9 females and 8 males) were treated with iodoacetic acid (IAA) and were enrolled in both temperament ($n = 8$ [4 females, 4 males]) and obstacle-course (OC; $n = 9$ [5 females, 4 males]) testing, whereas the remaining 5 (3 males and 2 females) were used as controls in the OC test. The pigs

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Table 1. Randomization into groups for obstacle course and temperament testing

Test	Group	Sample size (sex)	Age (wk)	Weight (kg)	Time of year
Obstacle course test	1	C, 2 (1 male, 1 female) IAA, 3 (1 male, 2 female)	9	25.8 ± 1.1	October–November
	2	C, 2 (1 male, 1 female) IAA, 3 (1 male, 2 female)	8	19.7 ± 2.7	February –March
	3	C, 1 (female) IAA, 3 (2 males, 1 female)	9	24.7 ± 1.9	April–May
Temperament testing	1	2 males, 2 females	12	47 ± 3.2	November– December
	2	2 males, 2 females	11	40.7 ± 2.5	May–June

All males were neutered.

were individually housed in 6-m² pens in the same room with visual and nose contact with their neighbors. The pigs were fed twice daily with a standard commercial pig diet (CESAC, Conselice, Ravenna, Italy), with free access to water. The 12:12-h light:dark cycle provided a minimum of 40 lx during the light period, and the temperature was maintained at 20 ± 2 °C. The room was sheltered from natural light, and experiments were performed from October through June (Table 1). Chains and wood were used as environmental enrichment;¹⁸ no bedding materials were used, to allow for easier sanitation. Every 2 d, the pigs each received a small amount of straw as rooting material.

IAA treatment. On the day of treatment, pigs were weighed and sedated with tiletamine–zolazepam (5 mg/kg IM; Zoletil, Virbac, Prague, Hungary). After 15 ± 3 min, general anesthesia was induced by using sevoflurane (SevoFlo, Esteve, Barcelona, Spain), administered by mask in a 1:1 oxygen:air mixture, and maintained by using the same halogenated agent.²⁹ Before IAA infusion, hematologic and biochemical analyses were performed. Briefly, blood samples were obtained from the femoral artery by using a 21-gauge butterfly needle and K₃EDTA anticoagulant and clot tubes (Terumo, Leuven, Belgium), for a total volume of withdrawn blood of approximately 5 mL, which was considered within safe blood-collection range and to have negligible effects for this species.³¹

IAA (CAS no. 64-69-7; lot nos. BCBQ7348V, BCBS2675V, and BCBT1628V, Sigma Chemical, St Louis, MO) solution was freshly prepared on the day of the administration by using sterile saline (0.9% NaCl; pH, 7.35 ± 0.05), to achieve a final concentration of 12 mg/mL. The 17 pigs treated received injections of 12 mg/kg into an auricular vein, whereas control pigs received intravenous injections of normal saline (NaCl 0.9%). The entire procedure lasted approximately 1 h and was performed on the same day for all animals in the same group (Table 1). All the animals recovered from anesthesia without complication. Two distinct behavioral tests were performed to assess vision in the pigs: the OC test and the temperament test. At the end of the 2 trials (day 44), pigs underwent ffERG to objectively confirm photoreceptor degeneration.

For ffERG, pigs were anesthetized by using the same protocol described for IAA treatment, and hematologic and biochemical analyses were performed as previously described. Two drops of oxybuprocaine hydrochloride (Novesina, Visufarma, Rome, Italy) were instilled on the surface of the cornea as a local anesthetic. A Barraquer blepharostat was used, and the eyes

were maintained in a central and stable position by 2 sutures. Corneal contact-lens electrodes (ERG-jet, Fabrial, La Chaux-de-Fonds, Switzerland) were used as active electrodes, whereas dermal needle electrodes (Bionen, Florence, Italy) were used as a reference under the ipsilateral eyelid. The ffERG stimuli (dark-adapted 0.01 ERG, dark-adapted 3.0 ERG, light-adapted 3.0 ERG) were produced by using a Ganzfeld dome, and the signals were acquired, amplified, and filtered (50-Hz low-pass FFT filter) by using WinAverager (Biomedica Mangoni, Pisa, Italy). The symbols – and + were assigned to indicate b-wave amplitudes of 50 µV or less and greater than 50 µV, respectively (Figure 1).

OC test. Nine pigs treated with IAA and 5 control animals were evaluated by using a previously described behavioral test¹⁷ with specific modifications. The pigs enrolled in the test were divided into 3 groups (Table 1), to facilitate their management. Animals were tested individually in the corridor of the piggery. The testing course was 10 m long and 2 m wide, with 10 solid obstacles (dimensions, 100 × 100 × 30 cm) placed at 1-m intervals along the right, left, or center of the course to obtain 4 different configurations (Figure 2). At the end of the course was a livestock scale, which they entered and received yogurt as a reward. The trial first required a training period of 2 wk before initiation of the eye treatment, and a maintenance period of 2 wk while photoreceptor degeneration developed (Figure 3 A). The test was repeated every Monday, Wednesday, and Friday during the training, trial, and maintenance periods; 6 replicates were recorded during the trial section. Pigs were always tested in the morning before feeding, in both light (150 lx) and dim (10 lx) conditions. Time (in seconds) to complete the course and the number of collisions with the obstacles (defined as any accidental hitting of one of the objects) were recorded concurrently by 2 independent observers. Collision was intended as any accidental hitting occurred. To reduce the chance that animals would memorize the obstacle positions, the configuration was changed every time the test was performed.

Temperament test. We evaluated a subset of 8 pigs by using a sequence of temperament tests (human approach test [HAT], novel object test [NOT], and open door test [ODT])⁹ before (day 11) and 3 wk after (day 37) IAA treatment. Animals were divided into 2 groups (Table 1), tests were completed consecutively and always in the order of HAT–NOT–ODT, and each animal was tested individually in its home pen. In the HAT, a person walked into the pen and stood still in the middle. In the NOT, a gray

Figure 1. Representative ff-ERG waves. (A) Light-adapted 3.0 ERG, b waves correspond to bipolar cell activation after cones respond to light stimuli. (B) Dark-adapted 0.01 ERG, b waves correspond to bipolar cell activation after rods respond to light stimuli. (C) Dark-adapted 3.0 ERG, b waves correspond to bipolar cell activation after all photoreceptors respond to light stimuli. Because dark-adapted ERG are recorded sequentially without further dark adaptation, only the 0.01 ERG is a fully dark-adapted response. Black waves correspond to a representative animal from group C; green waves to a representative of the IAA-treated group.

Figure 2. Obstacle course test. Graphic representation of the 4 obstacle configurations from above and side and front views of the obstacles.

plastic bucket was put in the middle of the pen. For both HAT and NOT, the time (in seconds) required for animals to make snout contact with the person or the bucket was recorded. In the ODT, the door of the pen was opened, and the time (in seconds) until animals left the pen was recorded. The same observer performed the testing on days 11 and 37 but did not have any other contact with the animals (that is, feeding or monitoring them) throughout the study.

Statistical analyses. Data were analyzed by using statistical software (version 15.11.0, MedCalc Software, Ostend, Belgium). All data for all variables were rejected for normality according to the Shapiro–Wilk test. Data for the continuous variable time from the OC test were analyzed by using the Mann–Whitney *U* test for independent samples, whereas data for the discrete variable number of obstacle collisions were analyzed by using the χ^2 test. Data from temperament testing were analyzed by using the Wilcoxon test for paired samples. Out-of-range data were automatically recognized and excluded from analyses. An outlier was defined as a value that was lower than the lower quartile minus 1.5 times the interquartile range or greater than the upper quartile plus 1.5 times the interquartile range (inner fences). An out-of-range value was defined as a value that was lower than the lower quartile minus 3 times the interquartile range or greater than the upper quartile plus 3 times the interquartile range (outer fences). The statistical unit for the OC test was the mean for time and median for obstacle collision among the 6 replicates for each animal (9 pigs IAA-treated, 5 controls). For temperament testing, the statistical unit was the animal (8 pigs each on days 11 and 37).

Results

Hematologic and biochemistry variables analyzed before and after IAA infusion were all within physiologic ranges for swine.³¹ The ffERG analyses confirmed the presence of induced photoreceptor degeneration in the pigs given IAA (Figure 1, Tables 2 and 3) and therefore the correct establishment of the pig model of photoreceptor degeneration.

The 14 animals enrolled in the OC test (Figure 4) were easily trained in 2 wk, such that after 3 d of training, all animals were accustomed to smells and noises during the test and paid no attention to the observers. The pigs were considered trained when they complete the course in less than 60 s without stopping to smell and explore the ambient environment. The maintenance period was necessary to give animals time to completely recover

A

B

Figure 3. Time line for (A) obstacle course test and (B) temperament testing. m, Monday; w, Wednesday; f, Friday; HAT, humane approach test; NOT, novel object test; ODT, open door test.

Table 2. Obstacle course test

	Time (s)		ffERG		
	Light	Dim	Dark-adapted 0.01 ERG	Dark-adapted 3.0 ERG*	Light-adapted 3.0 ERG
Control (<i>n</i> = 5)	18.11 ± 3.07 13.66–20.83	21.77 ± 3.89 16.33–27.00	+	+	+
IAA (<i>n</i> = 9)	37.37 ± 12.18 23.00–52.50	30.37 ± 1.91 28.00–32.66	—	—	—

Data are reported as mean ± 1 SD, followed by ranges. For ffERG results, – and + were assigned to indicate b wave amplitudes of ≤ 50 and >50 μV, respectively. Dark-adapted ERG were recorded sequentially, without further dark adaptation; therefore, only the 0.01 ERG is a fully dark-adapted response.

Table 3. Temperament testing

	HAT	NOT	ODT	ffERG		
				Dark-adapted 0.01 ERG	Dark-adapted 3.0 ERG	Light-adapted 3.0 ERG
Day 11	29.81 ± 58.50 1–220	8.37 ± 9.35 1–35	21.15 ± 42.11 1–169	+	+	+
Day 37	18.00 ± 39.43 1–153	8.56 ± 8.92 1–34	24.06 ± 20.64 3–69	—	—	—

Data are reported as mean ± 1 SD (*n* = 8), followed by ranges. For ffERG results, – and + were assigned to indicate b wave amplitudes of ≤ 50 and >50 μV, respectively. Dark-adapted ERG were recorded sequentially, without further dark adaptation; therefore, only the 0.01 ERG is a fully dark-adapted response.

from anesthesia and to obtain full photoreceptor degeneration.²⁰ The data, time, and the number of obstacle collisions (Tables 2 and 4) are those for the 2-wk trial period. Under both light and

dim conditions, the time to complete the trial was higher (*P* = 0.0095) in the IAA group than the control group but did not differ between lighting conditions within the same group. In addition,

Figure 4. Obstacle course test. The box plots indicate the median and interquartile range. (A) Time to complete the course under the light condition. (B) Time to complete the course under dim conditions. C, control group ($n = 5$); IAA, Iodoacetic Acid group $n = 9$. *, $P < 0.05$ (Mann-Whitney U test) between C and IAA groups; no differences were detected between test conditions.

Table 4. Frequency (%) of pigs that collided with obstacles in the OC test

Score	Light		Dim	
	C	IAA	C	IAA
0	100	0	100	0
1	0	75	0	0
2	0	25	0	25
3	0	0	0	50
4	0	0	0	25
Median score	0	1	0	3

The score represents the number of obstacle collisions. Control group, $n = 5$; IAA group, $n = 9$.

none of the control animals collided with any obstacles of the OC test, whereas pigs in the IAA group hit 1 to 4 obstacles (χ^2 test: light, $P = 0.0067$; dim, $P = 0.0186$). In light conditions, 75% of the IAA animals collided with 1 obstacle, whereas in dim conditions, 50% collided with 3 obstacles ($P = 0.0074$).

Data collected from the temperament test are reported in Table 4 and shown in Figure 5. The software recognized some data points as outliers; in all cases, the operator noted external interference (for example, food on the floor, sudden noise), and

Figure 5. Temperament Test. Box plot median interquartile range ($n = 8$). (A) Human approach test. (B) Novel object test. (C) Open door test. □, Out-of-range values; ○, outliers.

therefore these data were excluded from statistical analysis. The time to complete the 3 tests (HAT, NOT, ODT) on days 11 and 37 did not differ between the 2 groups.

Discussion

The study aimed to identify and validate a feasible and reproducible behavioral test to subjectively assess vision in a pig

model. We used a rapidly inducible pig model of photoreceptor degeneration obtained by the administration of 12 mg/kg IAA and confirmed degeneration through fERG evaluation. We investigated 2 distinct behavioral approaches to better understand how pigs react to vision impairment and to identify which testing scenario discriminates between normal and vision-impaired pigs. Pigs are diurnal animals with highly developed senses of smell and hearing, and these senses can add bias to a behavioral test designed to assess vision.^{3,7} Different behavioral tests, which were based on the ability to avoid obstacles, were used to assess vision in the IAA cat model and in the transgenic pig model of photoreceptor degeneration.^{11,17} In both cases, animals were not trained, and the efficacy of the test varied between animals regardless of their vision status. In our current study, the OC test was designed to include a 2-wk training period to accustom the animals to the trial environment, including new smells and noises. Because the animals achieved the desired outcome after only 3 d of training, we suppose that a shorter training period could be effective. The OC test, improved by this initial training period, was extremely sensitive in detecting differences between the control and IAA groups at 3 to 4 wk after treatment, under both light and dim conditions. Unlike in a previous study,¹¹ which found no correlation between low ERG and impaired navigational capacity, our pigs with fERG-confirmed photoreceptor degeneration differed statistically significantly from the control group in the OC test.

Our results demonstrate that pigs behaviorally react to vision impairment by increasing the number of collisions with obstacles and the time to complete the course. Initial training helps to avoid interferences inherent to the animal's individuality. Moreover, the OC test was run under different light conditions to discriminate between loss of rods (night vision) and cones (day vision). Compared with cones, rods are indeed more vulnerable to the metabolic stress produced by the administration of IAA, as demonstrated in rabbits,²² NHP,²¹ ground squirrels,⁶ and pigs.²⁷ We found that the number of obstacle collisions in the IAA group increased under dim conditions compared with light, suggesting that rods are more affected than cones by the IAA treatment. Moreover, the high variability of the IAA group in light conditions may suggest that cone function was partially preserved some animals. Our results are in accordance with those of previous authors, who found differences in fERG response between rods and cones at 5 wk after 12 mg/kg IAA treatment in domestic pigs.³² We, therefore, confirm that our OC test can detect differences in vision impairment due to specific photoreceptor damage. In addition, our pigs appeared to be excited to perform the OC test because the operator reported that they vocalized in the same way as when they were fed, and they showed a playful attitude when exiting their enclosures. Our test is based on food reward after a complex activity completion, acting as an environmental cognitive enrichment. These kinds of mental challenges are reported to have positive effects on animal welfare,³⁴ suggesting that the OC test might be used to improve the husbandry and management of pigs used in biomedical research.

We used temperament testing to investigate how pigs interacted with their environment before and after IAA treatment. The pigs enrolled in temperament trials were not tested in the OC test, given that the training likely would have altered the outcomes. Results from the HAT, NOT, and ODT did not differ between days 11 and 37. This outcome might indicate that the vision impairment does not alter the ability of the pigs to interact with humans and the environment under experimental husbandry conditions. The HAT, NOT and ODT have been used

in commercial pig husbandry to assess animal welfare and to correlate temperament-associated responses toward humans with performance.⁸ In our current study, the temperament testing was designed to investigate how vision impairment modifies an animal's reaction to humans (HAT) and objects (NOT) and their desire to explore (ODT). Temperament testing seems unable to detect vision impairment in laboratory pigs, but the results at day 11 suggest that the animals in our experimental facility were in appropriate husbandry conditions.⁹ Indeed, the average time to complete each of the temperament tests was very low (less than 30 s), compared with the previously allowed maximal time allowed for each test (300 s),⁹ thus indicating a low level of fear (toward both humans and new situations) and strong exploratory behavior. None of the 3 tasks (HAT, NOT and ODT) showed differences at day 37, suggesting that vision impairment, anesthesia, treatment, and manipulation by humans did not alter the individual behavioral characteristics. Previous studies reported a reduction in the time needed to complete the HAT and NOT tests when repeated over time,¹² probably due to a kind of habituation as animals became more acquainted with the test situation. The lack of time reduction detected in the present study could be attributed to the fact that the animals, when tested for the first time (day 11), were already accustomed to the environment and facility personnel. It is worth noting that favorable human-animal interactions and the ability to test the pigs in their home pen have both been described some of the most important factors to improve consistency in behavioral tests.¹⁵ In light of our findings, temperament testing was helpful in demonstrating that, under our experimental conditions, the behavioral characteristics of the tested animals were not modified by the experimental environment and manipulations, thus allowing the researcher to obtain clear and reproducible data from a behavioral test specifically designed to assess vision. Temperament tests might, therefore, be helpful in measuring stress and behavioral alteration in laboratory pigs undergoing experimental procedures, but more investigation is needed to confirm this thesis.

In conclusion, vision can be assessed in pigs by using the OC test. This test is able to detect early vision impairments after IAA treatment and to discriminate between the degeneration of rods compared with cones. This behavioral test is feasible and easy to reproduce, because it does not require any specific instrumentation or technical capabilities to execute. However, the training period that we conducted demonstrates the importance in avoiding bias due to individual animal characteristics. Overall, the OC test, as a refinement, helps to avoid repeated anesthesia and invasive procedures and acts as environmental cognitive enrichment, leading to increased animal welfare and robust data.

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