tvst

Methods

Ex-Vivo Method to Quantifiably Evaluate the Staining Effectiveness of Anterior Lens Capsule Dyes

Ivan Fernandez-Bueno[1–3], Ricardo Usategui-Martín[1–3], José Carlos Pastor[1–4], and Cristina Andrés-Iglesias[1]

[1] Instituto Universitario de Oftalmobiología Aplicada (IOBA), Retina Group, Universidad de Valladolid, Valladolid, Spain
[2] Centro en Red de Medicina Regenerativa y Terapia Celular de Castilla y León, Valladolid, Spain
[3] Red Temática de Investigación Cooperativa en Salud (RETICS), Oftared, Instituto de Salud Carlos III, Valladolid, Spain
[4] Department of Ophthalmology, Hospital Clínico Universitario de Valladolid, Valladolid, Spain

Correspondence: Ivan Fernandez-Bueno, Instituto Universitario de Oftalmobiología Aplicada (IOBA), Universidad de Valladolid, Campus Miguel Delibes, Paseo de Belén 17, Valladolid 47011, Spain. e-mail: ifernandezb@ioba.med.uva.es

Received: July 26, 2021
Accepted: November 19, 2021
Published: December 17, 2021

Keywords: vital dyes; lens capsule; ex-vivo; cataract

Citation: Fernandez-Bueno I, Usategui-Martín R, Pastor JC, Andrés-Iglesias C. Ex-vivo method to quantifiably evaluate the staining effectiveness of anterior lens capsule dyes. Transl Vis Sci Technol. 2021;10(14):17, https://doi.org/10.1167/tvst.10.14.17

Purpose: High frequency of cataracts and the requirements of new European Union regulations for medical devices require the availability of preclinical models to adequately evaluate anterior lens capsule dyes before their use in patients. Herein, we describe an ex vivo method to quantifiably evaluate the macroscopic and microscopic staining effectiveness of anterior lens capsule dyes using porcine eyes.

Methods: Commercially available trypan blue–based products or physiological saline solution (negative control) was injected into porcine eyes. Anterior pole and lens (after extraction) were macroscopically photographed, and the images were quantitatively analyzed. Lenses were histologically processed, and the staining intensity microscopically was semiquantified.

Results: Macroscopic evaluation of the anterior pole revealed bluish staining of the anterior capsule; however, this coloring cannot be macroscopically discerned after lens extraction. Quantitative image analyses showed significant ($P < 0.01$) staining of the lens capsule compared to the negative control, but not significant ($P > 0.05$) between the products tested. Quantitative analysis of dying on lens images could not be performed. Microscopic semiquantification of the capsule staining intensity allows us to appreciate differences between products.

Conclusions: The described method is a quick and useful tool for macroscopic evaluation by surgeons to choose an anterior capsule staining for use during everyday surgeries, and a more specific microscopic evaluation also allows us to determine the effectiveness and usefulness of these products.

Translational Relevance: This method satisfies preclinical effectiveness evaluations required by European Union regulations and complements the safety and toxicity evaluations that new products must guarantee before they enter the market and are used in clinical practice.

Introduction

Adequate capsulorrhexis is a critical step in cataract surgery.[1] Thus visualization of the capsular flap is critical in many scenarios, such as in cases with mature cataracts with no red fundus reflex, cases of hemophthalmia, traumatic cataracts, cataracts with a white or opalescent cortex, and cataracts in eyes with corneal opacities.[1–3] Moreover, poor capsular visualization increases the risk of complications at this surgical step, including radial capsular tears, vitreous loss, or dislocation of the intraocular lens.[2] Therefore one of the most important innovations over the past years has been the use of vital dyes to enhance anterior capsule visualization.[4]

Anterior lens capsule dyes should be sterile and biocompatible ophthalmic solutions for intraocular use. The ideal vital stain would have several characteristics, with the most important being its safety for

Copyright 2021 The Authors

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

Downloaded from hwmaint.iovs.org on 04/24/2024

translational vision science & technology

intraocular use. The stain also should reliably and selectively stain the anterior capsule and be able to be removed quickly from the eye to avoid potential adverse effects if the contact time is longer than what is necessary for the intervention. However, a suitable absorption profile may maximize the visualization of the stained tissue, which sometimes creates problems with potential tissue toxicity.[3]

Considering the high frequency of cataracts, the World Health Organization estimates that cataracts will develop in about 40 million people by 2025,[5] and continuous improvements in the intraocular dyes such as the ones used in the anterior capsule ex vivo methods to evaluate the staining effectiveness (efficacy) become highly relevant. Although previous studies have evaluated the staining ability of different dyes for use in the anterior capsule,[6–8] none have described an ex vivo standardized method that includes quantifiable evaluation of the macroscopic and microscopic staining effectiveness, which also complies with the requirements of the new European Union (EU) regulations for medical devices, as we described here.

Materials and Methods

Staining Procedure of the Anterior Lens Capsule

Porcine eyeballs (n = 15) were collected at a local slaughterhouse (Valladolid, Spain) from six- to 8-month-old castrated white pig breeds and transferred to the experimental operating room in refrigerated Dulbecco's modified Eagle medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The eyes were submerged in povidone-iodine solution (MEDA Pharma SAU, Madrid, Spain) for five minutes and washed in Dulbecco's modified Eagle medium for five minutes. The remaining extraocular tissues were removed using Westcott scissors (John Weiss, Milton Keynes, UK), and the eyeball was placed on an eye support pad (Phillips Studio Ltd., Bristol, UK) and fixed with pins. Each eye was carefully evaluated under a surgical microscope (Leica M220 F12 Surgical; Leica Microsystems, Wetzlar, Germany) to rule out possible ocular alterations, mainly lens opacity, that could interfere with the study.

Two commercially available lens capsule staining products (purified 0.06% trypan blue–based) were used, AJL Blue (AJL Ophthalmic S.A., Miñano, Spain) and Vision Blue (D.O.R.C., Zuidland, The Netherlands), a considered reference in clinical use and thus used as the positive control. A 30-gauge (G) cannula (Becton Dickinson, Franklin Lakes, NJ, USA) was connected to the syringe containing the intraocular dye, and the plunger was pushed out gently to facilitate the emergence of a small amount of product.

To avoid potential significant interference, the intraocular stains and their application in the porcine eyes were randomized using the Research Randomizer (www.randomizer.org).

A modified version of the under-air staining technique for the anterior capsule was applied.[9,10] A clear corneal incision was made using a 2.75-mm angled corneal knife (Alcon, Fort, Worth, TX, USA) under observation using a Leica M220 F12 surgical microscope. Using a 25-G cannula connected to a 5 mL syringe (both from Becton Dickinson), a large air bubble was injected through the incision and into the anterior chamber to clear the aqueous humor. The 30-G cannula connected to the anterior lens capsule staining syringe was inserted into the anterior chamber until the tip penetrated the air bubble and reached the center of the pupil so that the cannula was just between the anterior capsule and posterior corneal surface. At this location, the trypan blue–based dye was injected gently, and 0.05 mL were applied over the anterior capsule. The needle was removed, and the stain was left for 30 seconds. The remaining dye was removed by washing the anterior chamber with about 2 mL of physiological saline solution (PSS; B. Braun Medical SA, Barcelona, Spain). PSS instead of the intraocular dye was injected using the same procedure in five eyes and served as a negative control.

For macroscopic evaluation of the effectiveness of surface staining of the anterior lens capsule, the anterior pole was photographed with the Leica M220 F12 and standardized microscope settings (fixed illumination, zoom, retroillumination). The cornea was dissected with Wescott scissors, and the lens was extracted with a lens removal cannula (John Weiss). Then the lens was photographed with the Leica M220 F12 using standardized microscope settings. Finally, the lens was submerged in 10% formaldehyde (PanReac AppliChem, Darmstadt, Germany), for a minimum of 24 hours, for histologic processing and microscopic evaluation.

Macroscopic Evaluation

The anterior pole and lens images were analyzed using ImageJ software (version 1.53a; National Institute of Health, Bethesda, MD, USA), following a previously described method.[11] Thus color images were transformed to grayscale. The background in the channel of interest corresponding to the dye visualized in blue was subtracted, using the threshold value obtained from the negative controls (PSS). This value

translational vision science & technology

Downloaded from hwmaint.iovs.org on 04/24/2024

was applied to all images in the experiment. Finally, the mean values of the gray areas were measured in the corresponding channel and assigned as arbitrary units.

Microscopic Evaluation

After fixation, the lenses were cross-sectioned, processed by a HISTO-PRO 200 automatic tissue processor (Histo-Line Laboratories, Milan, Italy), and embedded in Paraplast-Plus paraffin (Merck KGaA, Darmstadt, Germany). Serial cuts 4 µm thick were made at different levels with a Microm HM340E microtome (Thermo Fisher Scientific), and the sections were stained with hematoxylin and eosin (Merck KGaA). The samples were examined and photographed by a Leica DM4000B light microscope equipped with a Leica DFC490 digital camera (Leica Microsystems). Finally, a comparative analysis of the acquired microscopic images was performed. Thus the staining intensity was semiquantified according to the following scale previously described[7]: no staining (negative control), baseline (positive control), less intense than baseline, or more intense than baseline.

Data Collection and Statistical Analysis

The generated data were collected in laboratory notebooks and databases created with Microsoft Office Excel 2016 software (Microsoft Corporation, Redmond, CA, USA). Quantitative variables were expressed as the mean ± standard deviation. The distribution of variables was analyzed using the Kolmogorov-Smirnov test. One-way analysis of variance was used to determine differences between quantitative variables. The statistical analyses were performed using SPSS software (version 24.0; IBM Corporation, Foster City, CA, USA), and $P < 0.05$ was considered statistically significant. Brightness and contrast of the images were minimally adjusted, and the final figures were composed with Pixelmator 3.8.2 Phoenix (Pixelmator Team, Vilnius, Lithuania).

Results

Staining Procedure of the Anterior Lens Capsule

None of the pig eyes presented notable ocular alterations and all presented a perfectly transparent lens. During the injection of the trypan blue–based dyes into the porcine eyes (Fig. 1), no significant differences were observed regarding the injection process, permanence in the anterior chamber, and surface staining effectiveness between the evaluated samples (AJL-Blue and Vision Blue). See Supplementary Video S1 for anterior capsule staining with trypan blue–based products in porcine eyes performance.

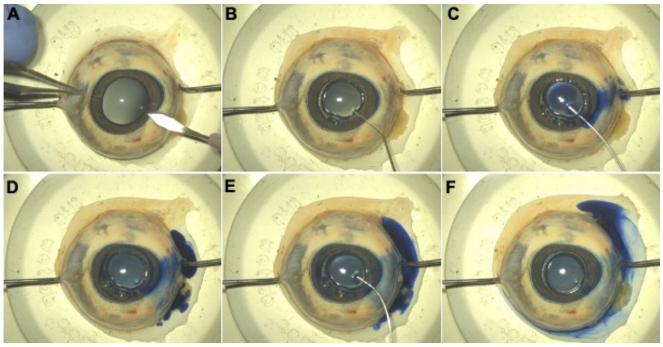

Figure 1. Anterior capsule staining with trypan blue–based products using a modified version of the under-air staining technique. (**A**) A clear corneal incision with a 2.75 mm angled knife; (**B**) a large air bubble injected into the anterior chamber to push out the aqueous humor; (**C**) dye gently injected at the center of the anterior chamber between the anterior capsule and the posterior corneal surface; (**D**) dye left 30 seconds; (**E**) remaining dye removed by washing the anterior chamber with PSS; and (**F**) lens anterior capsule final staining.

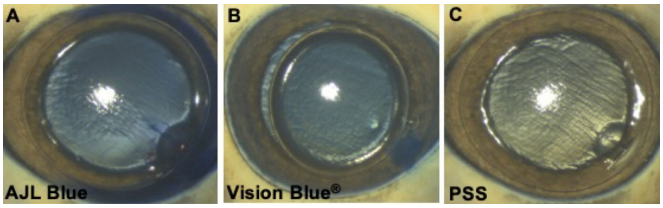

Figure 2. Macroscopic evaluation of the anterior pole after anterior capsule staining with trypan blue–based products. Commercially available products (**A**&**B**), including a reference in clinical use as the positive control (**B**) and PSS (**C**) as the negative control. The presence of a bluish stain is seen clearly on the anterior capsule (**A**&**B**).

Macroscopic Evaluation

Macroscopic evaluation of the anterior pole after anterior capsule staining with trypan blue–based products clearly showed the presence of a bluish staining of the anterior capsule (Figs. 2A–C). However, the presence of this bluish stain cannot be discerned on the anterior capsule after corneal dissection and lens extraction (Figs. 3A–C).

Quantitative analyses of the macroscopic staining effectiveness in the anterior pole images, including the cornea, anterior chamber, and lens, can be performed easily (user level) with the ImageJ software, a public domain image processing software that has become an important tool for measuring bioimages.[12] Image analyses showed significant (all cases, $P < 0.01$)

translational vision science & technology

Downloaded from hwmaint.iovs.org on 04/24/2024

Table. Quantification of the Macroscopic Staining Effectiveness in Anterior Pole Images Using ImageJ Software

Test item	Macroscopic Staining (Mean ± SD)	*P* Value
AJL Blue	16.42 ± 2.80 A.U.	= 0.932
Vision Blue	17.27 ± 3.65 A.U.	
PSS	0.1 ± 0.1 A.U.	vs. AJL Blue = 0.001 vs. Vision Blue < 0.001

SD, standard deviation; A.U., arbitrary units.

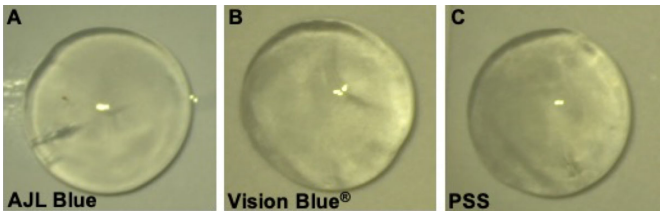

Figure 3. Macroscopic evaluation of the lens after anterior capsule staining with trypan blue–based products. Commercially available products (**A**&**B**), including a reference in clinical use as the positive control (**B**) and PSS (**C**) as the negative control. The presence of staining cannot be discerned on the anterior capsule after extraction of the lens (**A**&**B**).

staining of the anterior lens capsule compared to the negative control (PSS) (Fig. 2, Table). No significant differences (all cases, $P > 0.05$) between the stains tested were seen (Table 1). Nevertheless, the images of the lens after extraction could not be evaluated with this software because the presence/absence of dye could not be discerned by this computer tool.

Microscopic Evaluation

Microscopic evaluation of the porcine lens by histologic examination after hematoxylin and eosin counterstaining allowed clear differentiation of the lens capsule, lens epithelium, and lens fibers. Since PSS (negative control) is defined as "no staining" and the reference product as "baseline," the other tested dye showed a staining degree that was more intense than baseline (Fig. 4).

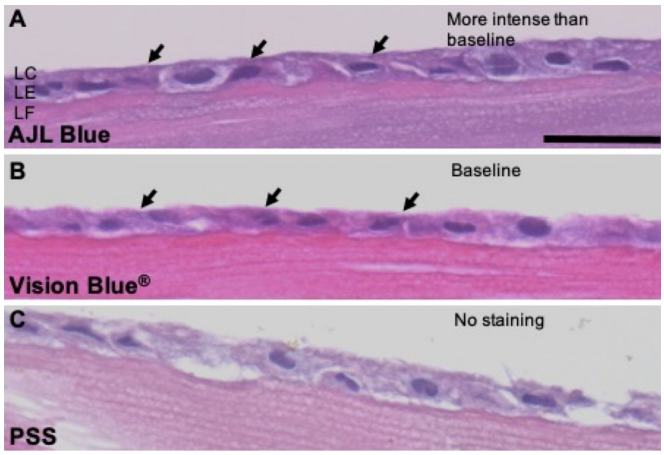

Figure 4. Microscopic evaluation of the porcine lens stained with trypan blue–based products. Commercially available products (**A**&**B**), including a reference in clinical use as the positive control (**B**) and PSS (**C**) as the negative control. Semiquantification of the capsule staining intensity was determined, with PSS defined as no staining and the gold standard product as the baseline. The other tested product showed a degree of staining more intense than the baseline. The *arrows* indicate the lens anterior capsule. *Scale bar*: 25 µm. LC, lens capsule; LE, lens epithelium; LF, lens fibers.

Discussion

Anterior lens capsule stains are ophthalmic solutions for intraocular use that facilitate visualization of the capsule flap in cataract surgeries. The ideal vital stain to enhance the anterior capsule visualization must be safe and effective for intraocular use. Therefore, the stain should be able to stain the anterior capsule reliably and selectively without damaging the surrounding tissues. Safety assessment of medical devices is included in the UNE-EN ISO 10993 and there are currently several in vitro models for the evaluation of intraocular vital dyes.[13–15] Furthermore, the new EU regulations for medical devices, EU directives 2017/745 and 2017/746, require scientific proof of the product's efficacy and mode of action and verification and validation studies of its effectiveness through preclinical and clinical data. Thus considering the EU regulations, the high frequency of cataracts, and the continuous improvement of the intraocular dyes, we described a new preclinical method to quantifiably evaluate the staining effectiveness of anterior lens capsule dyes in porcine eyes ex vivo.

The modified version of the under-air staining technique of the anterior chamber described here was simple, cost-effective, and achieved consistent, uniform, and reproducible staining in porcine eyes ex-vivo. Porcine eyes are specifically chosen for ex-vivo experiments because of availability, low price, and size

translational vision science & technology

Downloaded from hwmaint.iovs.org on 04/24/2024

similar to human eyes, and differ only in the greater corneal thickness and tissue hardness, which facilitates the performance of surgical procedures.[16,17] Furthermore, porcine eyes share many similarities with human eyes in the morphology and physiology of the anterior chamber.[16–18] The under-air staining technique,[9,10] during which viscoelastic substances were not used, prevents the air from exiting during dye injection and results in increased anterior chamber stability. Moreover, this technique allows selective staining of the anterior capsule and avoids excessive dye build-up in the anterior chamber.[9]

The first approach, macroscopic evaluation of the anterior pole, resembles the surgeon's view of a patient's surgical procedure, with the presence of the cornea, anterior chamber, and anterior surface of the lens, and therefore it is more similar to actual clinical practice. Thus the current results indicated that the staining effectiveness of the tested dyes using macroscopic images of the anterior pole can be assessed. Nevertheless, macroscopic evaluation of the lens after extraction could not be assessed by the described image processing protocol. The first step when analyzing and quantifying the color intensity by image processing software is separating the color layers in the images (split channels). The low intensity of blue detected by the software compared with the rest of the image in the extracted lens images did not allow the program to adequately split channels and thus measure the staining effectiveness of the anterior capsule dyes. Furthermore, it is also noteworthy that the bluish staining was not discernible under the surgical microscope after lens extraction. Concerning these findings, we have evaluated anterior capsule staining after dissection and removal of the cornea, showing macroscopically a bluish staining and statistically significant staining compared to the negative control on ImageJ analysis (data not shown). Thus we discard any remaining dye in the anterior chamber (excess staining was removed with abundant PSS) nor potential staining of the corneal endothelium (no bluish coloration was detected in the cornea during its dissection) as the origin of the bluish staining observed in the whole eye and not discerned in the lens. Furthermore, studies in humans have shown that under routine use trypan blue remains in the anterior chamber, it does not stain the corneal endothelium.[19] In our opinion, positioning the lens flat on a light background does not provide images with enough contrast for the image processing software to discern the blue channel. Nevertheless, for future experiments, we propose transillumination and advanced image capture techniques to reveal lens staining and that allows us to measure and analyze quantitatively.

The second approach, microscopic evaluation of the porcine lens, allows clear differentiation of the lens histologic structure. Although the thickness of the porcine anterior lens capsule is thinner than that in humans,[20,21] semiquantification of the capsule staining intensity can be determined. Thus our current results indicate that an effectiveness score, such as that previously described by Wilińska et al. (2019),[7] can be assigned to the tested dyes using microscopic studies of the lens.

In conclusion, the lens anterior capsule staining ability of intraocular dyes can be determined easily in an ex vivo standardized method in porcine eyes. Macroscopic evaluation can be a quick and useful method for surgeons to choose a product for use during everyday surgeries, and a more specific microscopic evaluation determines the actual effectiveness and usefulness of new products. Further, the method described satisfies the effectiveness evaluations required by the EU regulations on medical devices (EU 2017/745 and 2017/746 directives) and complements the appropriate safety and toxicity evaluations that new products must satisfy before they become commercially available.

Acknowledgments

The authors are grateful to the staff of the Justino Gutierrez S.L. Slaughterhouse (Valladolid, Spain) for providing the porcine eye globes used in this work. We also thank Lynda Charters from Medical International (Framingham, MA, USA) for her assistance in the final editing and preparation of this manuscript.

Disclosure: **I. Fernandez-Bueno,** ISO 16672:2020 Ophthalmic Implants–Ocular Endotamponades (S); **R. Usategui-Martín,** None; **J.C. Pastor,** ISO 16672:2020 Ophthalmic Implants–Ocular Endotamponades (S); **C. Andrés-Iglesias,** AJL Ophthalmic S.A. (F), ISO 16672:2020 Ophthalmic Implants–Ocular Endotamponades (S)

References

1. Jacobs DS, Cox TA, Wagoner MD, Ariyasu RG, Karp CL. Capsule staining as an adjunct to cataract surgery. A report from the American Academy of Ophthalmology. *Ophthalmology*. 2006;113:707–713.
2. Efremov YM, Bakhchieva NA, Shavkuta BS, et al. Mechanical properties of anterior lens capsule

translational vision science & technology

Downloaded from hwmaint.iovs.org on 04/24/2024

assessed with AFM and nanoindenter in relation to human aging, pseudoexfoliation syndrome, and trypan blue staining. *J Mech Behav Biomed Mater*. 2020;112:104081.
3. Jackson TL, Griffin L, Vote B, Hillenkamp J, Marshall J. An experimental method for testing novel retinal vital stains. *Exp Eye Res*. 2005;81:446–454.
4. Morales MC, Freire V, Asumendi A, et al. Comparative effects of six intraocular vital dyes on retinal pigment epithelial cell. *Invest Ophthalmol Vis Sci*. 2010;51:6018–6029.
5. Wang W, Yan W, Fotis K, et al. Cataract surgical rate and socioeconomics: A global study. *Invest Ophthalmol Vis Sci*. 2016;57:5872–5881.
6. Sinumol S, Jayalakshmy PS, Sreedharan T, Unni MO, Narayan S, Sujatha N. Effect of intraoperative trypan blue on lens epithelial cells—Histomorphological analysis. *Saudi J Ophthalmol*. 2019;33:18–23.
7. Wilińska J, Mocanu B, Awad D, et al. New stains for anterior capsule surgery. *J Cataract Refract Surg*. 2019;45:213–218.
8. Yetik H, Devranoglu K, Ozkan S. Determining the lowest trypan blue concentration that satisfactorily stains the anterior capsule. *J Cataract Refract Surg*. 2002;28:988–991.
9. Giammaria D, Giannotti M, Scopelliti A, Pellegrini G, Giannotti B. Under-air staining of the anterior capsule using Trypan blue with a 30 G needle. *Clin Ophthalmol*. 2013;7:233–235.
10. Om Parkash R, Mahajan S, Om Parkash T. Modified 30 G needle trypan blue staining technique under air for a uniform and consistent anterior capsule staining. *Clin Ophthalmol*. 2017;11:1651–1656.
11. Soriano-Romaní L, Contreras-Ruiz L, García-Posadas L, López-García A, Masli S, Diebold Y. Inflammatory cytokine-mediated regulation of thrombospondin-1 and CD36 in conjunctival cells. *J Ocul Pharmacol Ther*. 2015;31:419–428.
12. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9:671–675.
13. Kim SY, Yang J, Lee YC. Safety of moxifloxacin and voriconazole in corneal storage media on porcine corneal endothelial cells. *J Ocul Pharmacol Ther*. 2010;26:315–318.
14. Ayaki M, Shimada K, Yaguchi S, Koide R, Iwasawa A. Corneal and conjunctival toxicity of disinfectants—Assessing safety for use with ophthalmic surgical instruments. *Regul Toxicol Pharmacol*. 2007;48:292–295.
15. van Dooren BT, Beekhuis WH, Pels E. Biocompatibility of trypan blue with human corneal cells. *Arch Ophthalmol.* 2004;122:736–742.
16. Middleton S. Porcine ophthalmology. *Vet Clin North Am Food Anim Pract*. 2010;26:557–572.
17. Sanchez I, Martin R, Ussa F, Fernandez-Bueno I. The parameters of the porcine eyeball. *Graefes Arch Clin Exp Ophthalmol*. 2011;249:475–482.
18. McMenamin PG, Steptoe RJ. Normal anatomy of the aqueous humour outflow system in the domestic pig eye. *J Anat*. 1991;178:65–77.
19. Abdelmotaal H, Abdelazeem K, Hussein MS, Omar AF, Ibrahim W. Safety of trypan blue capsule staining to corneal endothelium in patients with diabetic retinopathy. *J Ophthalmol*. 2019;2019:4018739.
20. Sueiras VM, Moy VT, Ziebarth NM. Lens capsule structure assessed with atomic force microscopy. *Mol Vis*. 2015;21:316.
21. Krag S, Andreassen TT. Biomechanical measurements of the porcine lens capsule. *Exp Eye Res*. 1996;62:253–260.

Supplementary Material

Supplementary Video S1. Ex vivo anterior capsule staining with trypan blue–based products in porcine eyes. A modified version of the under-air staining technique.

translational vision science & technology

Downloaded from hwmaint.iovs.org on 04/24/2024