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A technical protocol for an experimental *ex vivo* model using arterially perfused porcine eyes

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ABSTRACT

Ex vivo ocular perfused models have been described in the past and were applied in different mammalian species as platforms to test drug delivery systems and surgical techniques. However, reproduction of those methods is challenging because extensive and precise description of the protocols used is lacking. In this technical paper we provide a detailed description of all the steps to be followed from the enucleation of porcine eyes to cannulation of the ophthalmic artery and perfusion. This model can contribute to the reduction of use of living animals in ophthalmology research, whereas as opposed to *in vitro* models, it preserves tissue complexity and integrity.

1. Introduction

The unique anatomy and presence of physical and biological barriers between the different ocular tissues make drug delivery to the posterior segment of the eye one of the greatest challenges in ophthalmology. High elimination rates, tight membrane structures, high blood flow in the choroid, and immunological responses are some of the factors that clearly indicate the need for the development of novel drug delivery technologies to target the posterior eye segment (Díaz-Coránguez et al., 2017; Del Amo et al., 2017).

Efficacy and safety testing of novel drug candidates are mostly performed by using *in vitro* cell models or animal models *in vivo*. *In vitro* testing suffers from over-simplification of the complicated ocular anatomy to only one or a few cell layers and therefore very often does not take into consideration the action of the ocular barriers. Compared to the human eye, posterior ocular physiology might vary between different animal species in terms of vasculature, the cellular anatomy of the retina, the position of the optic nerve head and the area centralis (Vézina, 2012). Despite these anatomic differences, animal models have been extensively used in ophthalmology research because they can provide an advantage over *in vitro* testing: preservation of ocular tissue integration and functionality, which consist important requirements in pharmacokinetic studies.

However, recently a strong societal drive for reduction and even elimination of the use of living animals in pharmaceutical testing has arisen. In 1992, W. Russel and L. Burch described in their book "The principles of humane experimental techniques" multiple guidelines for more ethical use of animals in experimental research (Russell and Burch, 1992). These principles are also known as "The 3Rs", which abbreviates the words "Reduction", "Replacement" and "Refinement". In order to implement the principles of the 3Rs, various organizations and publications intend to provide advice and description of laboratory methods that can improve the welfare of laboratory animals and make the experiments more reproducible (Singh, 2012; De Boo and Hendriksen, 2005). Apart from the ethical aspect, *in vivo* animal testing requires intensive and detailed efforts to prepare documentation for the animal ethics committee and high financial resources. As opposed to animal testing, an alternative that is in line with the principles of the 3Rs and can be particularly applied in ocular drug delivery research, is the use of *ex vivo* eye models. Fresh enucleated eyes can be obtained by animals that are sacrificed for other purposes. These can be either tissues collected after the completion of *in vivo* animal experiments as material that would be otherwise considered as waste, or as slaughterhouse material.

Extracorporeal arterially perfused eye models were introduced in the past and used to study the ocular disposition of dyes and drugs (Abarca et al., 2013; Koeberle et al., 2006; Blue, 1997; Niemeyer, 2001), and to validate novel instrumentation and surgical techniques

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(Van Overdam et al., 2018; Chen et al., 2011; Townsend et al., 2006). For these models, eyes from different mammalian species were used, with feline and bovine eyes being the most frequently encountered. In 1970 Gouras and Hoff (1970) developed an extracorporeal perfused system where retinal function and response of the optic nerve were analyzed using cat eyes. Later, similar *ex vivo* systems were utilized to evaluate the retinal response as a function of perfusate flow rate (Niemeyer, 1973), and the variation of oxygen tension gradients as a function of the distance from the retinal vessels (Alder et al., 1986). Furthermore, the feline eye was used as a model to study the effect of administration of pharmaceutical compounds on the retinal function (Sandberg et al., 1987; Thoreson and Purple, 1989). Similarly, electroretinographic activity was measured using *ex vivo* bovine eyes, while the influence of anoxia and hypothermia on the retinal function was studied (Tazawa and Seaman, 1972). Coo *et al.* examined the response of the extracorporeal bovine eye in normothermic conditions and performed a viability study to determine tissue functionality under continuous perfusion (De Coo et al., 1993). However, in terms of resemblance of the anatomy of the human eye feline and bovine eyes are not the best choice.

In this report we describe in detail the preparation method of extracorporeally perfused porcine eyes as these show numerous similarities with the human eye with respect to the anatomy of the optic nerve and retinal vasculature, elasticity and size (Coile and O'Keefe, 1988; Simoens et al., 1992; Vestre, 1984). This method is based on cannulation of the ophthalmic artery, which originates from the internal carotid artery. The cannulation point was chosen to be just proximal of the branch of the ophthalmic artery to the anterior and posterior ciliary arteries and the central retinal artery, thus allowing for holistic perfusion of the organ (both the anterior and posterior segments). An essential requirement for successful cannulation is the preservation of the ophthalmic artery attached to the enucleated tissue. Since the ophthalmic artery runs parallel to the optic nerve, a prerequisite is preservation of at least 4 cm of the optic nerve attached to the globe. Therefore, we provide the reader with a step-by-step protocol, images and detailed videos describing how to enucleate, dissect, cannulate the ophthalmic artery and perfuse the porcine eye.

The protocol provided will allow other researchers to reproduce this *ex vivo* model, utilizing it as an experimental platform that can be used for the study of disposition of novel drugs or to validate novel surgical instrumentation/techniques. Particularly in the case of drug delivery testing, perfusion of the entire eye gives the advantage to this model being not only limited in testing of intravenously administered therapeutics, but also expanded in other routes of administration (e.g. intravitreal, sub-retinal, etc.).

2. Materials and Supplies

The name of suppliers and catalogue numbers of the materials and equipment used for the preparation of the system are given in Table 1, including comments on the characteristics and the quantity needed. To ensure more clarity, the materials that require specific characteristics are marked with asterisk (*), and images can be found in the supplementary information (Table S1). The preparation of the model is performed under a binocular operational microscope, with magnification that can vary between eyes from different pigs, but minimum 10×.

3. Detailed methods

In this section a detailed description of the procedures followed for enucleation, transportation, dissection and cannulation of the ophthalmic artery, and testing of perfusion is given. For more details and visual clarity, videos and images recorded during the implementation of the protocol are included. The videos are entitled as follows:

- Localization and isolation of the ophthalmic artery
- Preparation of the ophthalmic artery and cannulation
- Testing of cannulation and perfusion of the retina

From now on the videos are referred to as Video 1, 2 and 3, respectively.

Supplementary videos related to this article can be found at https://doi.org/10.1016/j.exer.2019.02.003

3.1. Enucleation and transportation of porcine eyes

Tissue preservation in good conditions requires fast eye enucleation. To avoid blood clotting, it is preferred to enucleate the eyes directly after animal termination, immediately after dissection of the carotid arteries for bleeding. Avoid enucleation of eyes from slaughtered animals that have been exposed to heating or immersed in a high-temperature water bath for skin cleaning and hair removal purposes, since this can cause heat-induced damage of the tissue. It is recommended that eye enucleation is performed not more than 1 h after animal termination.

When dissecting the eye, an intact section of adequate length of the optic nerve (≥4 cm) should be preserved. To ensure stability and control of the ocular area during enucleation use two towel forceps. Use one of the towel forceps to close the eyelids of the eye that will be enucleated and clamp them together (Fig. 1a and b). Use the other towel forceps to fix the ear at the enucleation side together with the neck skin (Fig. 1c). This will ensure more free space during enucleation. Incise the skin with a scalpel at the nasal and temporal side of the eye (Fig. 1d, solid lines). Use one hand to hold the towel forceps that clamps the eyelids, and the other hand to incise the skin and periocular tissues by moving the scalpel parallel to the orbital rim (Fig. 1e, dashed line). At this point, the eyelids are still attached to the anterior part of the eye. Apply moderate pressure on the forceps clamping the eyelids to pull the eye slightly out of the orbital cavity. Locate the optic canal in the sphenoid bone by touch and dissect the optic nerve as deep as possible. This latter step represents the most crucial of all the enucleation steps, because it must ensure that the length of the optic nerve (distance between the posterior side of the eye and the dissection point) is at least 4 cm. Dissect any other periocular tissues that surround the eye inside the orbital cavity (muscles, connective tissues) and pull the eye entirely out of the orbital cavity. Remove the attached periocular tissues from the anterior part (eyelids, lacrimal gland, etc.). Immerse the eye in heparinized saline (50 units/ml) stored at 0 °C (melting ice) to decrease the metabolic activity of the tissues. Repeat the procedure to collect more eyes.

Based on our experience, even if only one eye is required for the experiment, six to eight eyes should be enucleated on the same day. Especially at the first stages of the training on this method, approximately 50% of the eyes collected are damaged and not appropriate for perfusion. More details on how to recognize the eyes that are damaged can be found in Section 4, "Potential pitfalls, trouble shooting and discussion". The time interval between harvesting and preparation of the *ex vivo* model has to be as short as possible and should never exceed 6 h.

Transportation of slaughterhouse material to university laboratories in the Netherlands requires yearly registration of a transportation document to the "Netherlands food and consumer product safety authority (NVWA)". In that document the researcher is obliged to report the type and biosafety level of the animal tissue transported, the average amount of tissue expected to be transported each time, the name and contact details of the transporter, and the address, contact details and license number of the abattoir. This document should always be available for presentation in case of inspection. In addition, records of the number of enucleated eyes obtained each time should be kept. It is important to note that regulations concerning the transportation of slaughterhouse material might vary between different countries.

Table 1
Names, sources and catalogue numbers of the materials needed for the enucleation and cannulation of the eyes. The materials marked with asterisk require specific characteristics.

Material name	Source	Catalogue number	Comments
Towel forceps (*)	Karl Storz, Germany	796011	Length: 11 cm, quantity: 2
Scalpel	Swan-Morton, UK	0934	Stainless steel, handle number 4
Surgical scalpel blades	Swan-Morton, UK	0311	Blade number 24, stainless steel, quantity: 2
Micro preparation forceps (*)	VWR, Netherlands	232–1917	Fine tip, length: 105 mm, quantity: 2
Micro-forceps (*)	Lawton Medizintechnik, Germany	09–0957	Superfine points, length: 11 cm, quantity: 2
Curved forceps	VWR, Netherlands	232–0106	Length: 105 mm, quantity: 2
Microscopy scissors	VWR, Netherlands	233–2123	Length: 100 mm, quantity: 1
Spring scissors (*)	Harvard Apparatus, MA, USA	728503	Vannas micro-dissecting (Eye) scissors, length: 8.5 cm, straight, spring action
Straight/dissecting forceps (*)	VWR, Netherlands	82027–388	Stainless steel, length: 114 mm quantity: 1. The opening distance of the tip should be maximum 1 cm. If it is more than 1 cm, use tape to shorten the distance of the opening.
Suture	Harvard Apparatus, MA, USA	72–3336	Suture material PolyGlycolic undyed brd., length: 70 cm, 5-0. Reverse cutting, C6, length: 19 mm.
Green plastic eye holder (*)	R2Pro, Netherlands	Custom made	Diameter of cup: 2.3 cm outer diameter: 6 cm, height: 2.2 cm, quantity: 1
Black sponge eye holder (*)	R2Pro, Netherlands	Custom made	The bottom side consists by four channels with soft glue. Diameter of the cup: 3.3 cm, diameter of the cannula opening: 1.5 cm, height: 3 cm, size of channels: 7 cm × 2.1 cm, quantity: 1
Cannula (*)	Smiths Medical 800/100/100, Netherlands	02	Fine Bore LDPE (polyethylene) tubing, inner diameter: 0.28 mm, outer diameter: 0.61 mm
Needle 19 G (*)	Any		With bended tip, quantity: 1
Needle 24 G (*)	Any		Cut the sharp tip of the needle and connect with the cannula, quantity: 1
Syringe 1 ml	Any		Quantity: 2
Syringe 50 ml	Any		Quantity: 1
Contact lens (*)	Oculenti BV, Netherlands	PR98080518	Bandage lens, hydrolent 67
Coupling gel (*)	Bausch & Lomb, Belgium	8000000027	EYEFILL high dispersive (H.D.), 2% HPMC in physiological saline solution
Heparin	LEO Pharma, Netherlands	RVG 01372	Heparin natrium, 5.000 U.I./ml
Sodium Chloride (Normal saline)	Any		NaCl 0.9%
Tissue adhesive (*)	B. Braun, Germany	1050052	Histoacryl blue topical skin adhesive
Gelofusine	B. Braun, Germany	150347642	Succinylated gelatin 4%
Fluorescein eye drops	Bausch & Lomb, Belgium	02406497	Minims 0.5 ml, fluorescein sodium 2%, eye drops solution
Syringe pump	Fresenius vial, France	17522534	Pilot delta, quantity: 1
Cotton buds	Any		Quantity: minimum 4
Pins	Any		Quantity: minimum 8

3.2. Dissection and cannulation of the ophthalmic artery

When ready to start the dissection and cannulation procedures, compare all the eyes that have been obtained and start with the one that has a considerable length of the optic nerve preserved, as well as the surrounding connective tissue. Locate the eye into the rigid plastic holder with the anterior part (i.e. cornea) touching the holder cup and the optic nerve facing upwards (Fig. 2).

3.2.1. Localization and isolation of the ophthalmic artery

A schematic representation of the main arteries within the ocular vascular network is shown in Fig. 3. The ophthalmic artery (diameter 0.8 mm) can be cannulated just proximal of the branching point of the short and long posterior ciliary arteries, and the central retinal artery, as shown with the black arrow in Fig. 3. Use the two micro-preparation forceps with fine tip to carefully remove the connective and muscle tissues, and isolate the ophthalmic artery (Video 1, 00:08–01:03). The artery wall appears white in color, slightly transparent and usually contains remaining native blood (Video 1, 01:04–01:15). Once the ophthalmic artery is isolated, remove any remaining surrounding tissue with the use of two micro-forceps, until the blood vessel is entirely stripped (Fig. 4 and Video 1, 01:16–02:25). To prevent vessel damage due to dryness, moisture the tissue at regular intervals (especially when the artery is stripped) with a cotton bud that is wetted with saline.

3.2.2. Preparation of the ophthalmic artery and cannulation

Keep the artery stretched without applying excessive tension by using a straight forceps (Fig. 5a and Video 2, 00:12–00:27). Make a small incision on the longitudinal axis of the blood vessel using spring scissors (Fig. 5b and Video 2, 00:28–00:33), and loosen the incision with the bended-tip needle (19 G), which is connected to a 1 ml empty syringe. If the incision is not large enough for the needle tip to penetrate into the vessel, make the cut larger (Video 2, 00:34–01:20). Subsequently, lift the upper vessel wall by pulling the syringe upwards, loosen the blood vessel wall and gently remove the needle. Fill the second 1 ml syringe with saline, remove air bubbles, and connect it to the cannula. With one hand, using the bended-tip needle, lift the upper wall of the vessel slightly. With the other hand, keep the cannula near the front end with curved forceps, oriented parallel to the artery, and simultaneously insert it into the vessel (Video 2, 01:21–01:27). The tip of the cannula must be pointing towards the direction of the optic nerve that enters the eye (Fig. 5c). Using the second curved forceps, gently press the blood vessel walls on the cannula and at the same time insert the cannula tube further in the vessel. The cannula should be placed 0.5–1 cm in the artery (Fig. 5c and Video 2, 01:28–01:38). Make a tight, double knot to ensure that the cannula is stably fixed in the blood vessel (Fig. 5d and Video 2, 01:39–01:43). Release the vessel from the straight forceps and add 3–4 drops of tissue adhesive (Fig. 5e and Video 2, 01:44–01:56). Wait for 1 min for the tissue adhesive to dry. This will ensure that the connection is tight enough and any leakage during perfusion is prevented.

3.2.3. Testing of cannulation and perfusion of the eye

During perfusion the eye should be kept in humid environment e.g. inside a custom-made, sponge eye holder wetted with saline (Fig. 6a). Disconnect the cannula from the syringe, pass it through the holder hole, and place the holder on top of the eye so that the posterior segment of the eye is inside the cup of the holder. After this step the eye is positioned between the two holders (the green plastic holder is below and the black sponge holder is on top). The cannula should be placed parallel to one of the four channels of the holder. Apply slight pressure on the cannula to ensure that it is fixated in the holder channel

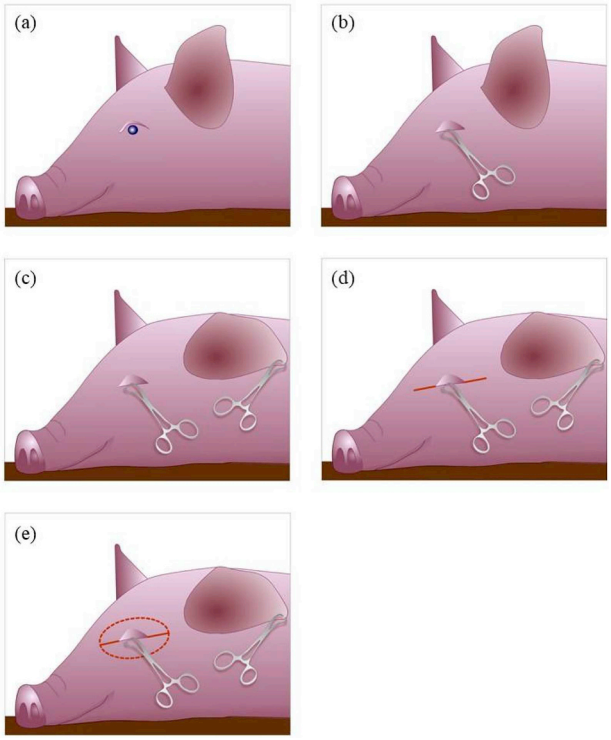

Fig. 1. Schematic representation of the incision steps followed for the enucleation of the eye. Use a towel forceps to close the eyelids and clamp them together (a, b). Use the second forceps to clamp the ear at the enucleation side with the neck skin to ensure more free space (c). By using a scalpel two incisions are made towards the nasal and temporal side of the eye (d) and subsequently the skin and periocular tissues are incised parallel to the orbital rim (e).

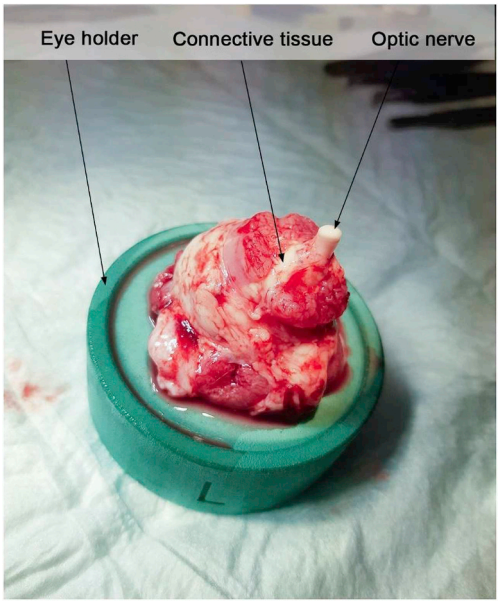

Fig. 2. Enucleated porcine eye positioned inside the plastic eye holder with the anterior segment (e.g. cornea) facing downwards. The optic nerve is approximately 4 cm in length. The ophthalmic artery is normally located into the connective tissue indicated by the middle arrow.

(Fig. 6a). Carefully flip the holder and the eye so that the anterior part is facing upwards (Fig. 6b). Insert pins to fix the periocular tissues on the sponge holder, having the cornea aligned in the center of the holder. To further improve the microscopic examination of the retinal blood vessels during perfusion, a contact lens is used to increase the magnification. Add a few drops of coupling gel on top of the cornea and place the contact lens on top of it. Avoid entrapping air bubbles between the gel and the lens. Adjust the microscope focus to have a clear image of the retinal blood vessels and the optic disc (Fig. 6b).

Successful cannulation can be confirmed by perfusing the eye with a dye, e.g. fluorescein. To maintain the volume of blood in the blood vessels and prevent the formation of blood clots, a perfusion test solution containing gelofusine and heparin is used. For the preparation of the perfusion test solution add 0.18 ml fluorescein eye drops and 5000 units of heparin in 100 ml of gelofusine. Fill the 50 ml syringe with the test solution, eliminate air bubbles and connect it to the syringe pump. Disconnect the cannula from the 1 ml syringe and connect it to the 50 ml syringe. Connect the syringe to the pump and initiate perfusion of the test solution at a flow rate of 0.1 ml/min. After a few seconds the retinal arteries can be seen filled with the dye if the cannulation is successful. While the arteries are filled, move the contact lens and scan the entire retina. A few seconds later, the retinal veins will be filled with the dye too. Check the cannulation point for leakage. If there is no leakage at the cannulated area and the vessels are normally filled with dye, continue perfusing with the test solution, or any other solution that is desired for circulation.

An example of how to test the cannulation and initiate perfusion is given in Video 3. In this example cannulation was initially tested with perfusion with saline. Note that at the start of the injection, first the native blood that is present in the retinal vessels is rinsed out (Video 3, 00:14–00:34). Subsequently, perfusion test solution containing fluorescein was perfused. Note that the dye first arrives in the retinal arteries, which appear smaller in diameter, followed by perfusion of the retinal veins (Video 3, 00:39–00:54).

4. Discussion, potential pitfalls and trouble shooting

In general, preparation of this system is a process that requires training in many aspects: the correct enucleation of the eyes, isolation of the ophthalmic artery, cleaning the ophthalmic artery from surrounding tissues, and cannulation. The target blood vessel for cannulation is the ophthalmic artery, which emerges from the internal carotid artery, and later branches to the ocular and orbital group of arteries (Fig. 3). The ophthalmic artery runs parallel to the optic nerve, and lies proximal of the branching point of the short and long posterior ciliary arteries, and the central retinal artery. Enucleation of eyes should be performed only by a person who has been trained, since a sufficient length of the optic nerve is a crucial prerequisite for preservation of the arterial part that consists the ophthalmic artery.

In the beginning of the training, the cannulation steps can be practiced using eyes that are not necessarily obtained from a slaughterhouse, for instance from laboratory animals that have been euthanized for other research purposes. The eyes used for training can be either fresh or non-fresh, but the tissues must always be at room temperature when the procedure starts. However, perfusion of the test solution most likely will not be successful on freeze-stored eyes because of blood clots that might block the blood vascular network. When the researcher feels comfortable with isolation of the ophthalmic artery on non-fresh eyes, he/she should immediately start practicing on fresh eyes, since the texture and the color of fresh tissues differ from tissues that underwent freezing and defrost procedures (fresh tissues are softer and color is more reddish since blood is still present). The procedure of enucleation in an abattoir directly after animal termination can be stressful and requires handling in a limited amount of time. Therefore, it is recommended to collect as many eyes as possible, since most likely not all of them will be suitable for cannulation. We recommend to collect at least three times the number of eyes needed for experimentation, especially at the initial training stages.

In the literature there is a discrepancy regarding the duration of time between animal termination and enucleation of the eyes. In the study of Abarca *et al.* porcine eyes were enucleated after animal

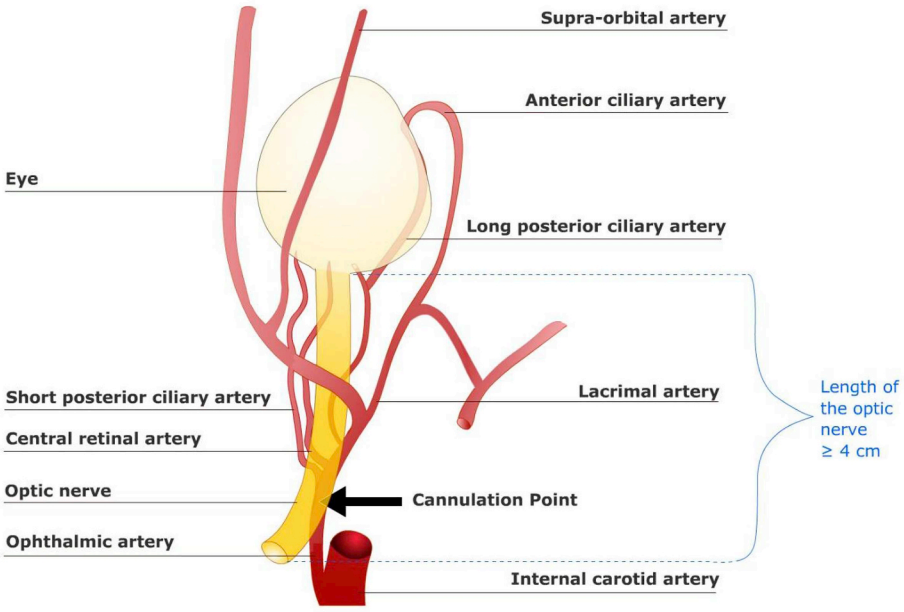

Fig. 3. Schematic representation of the ophthalmic artery and its branches originating from the internal carotid artery. The point where dissection and cannulation is performed is just proximal of the branching point of the short and long posterior ciliary arteries, and the central retinal artery (black arrow). Yellow: optic nerve, white: eye, red: arteries. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

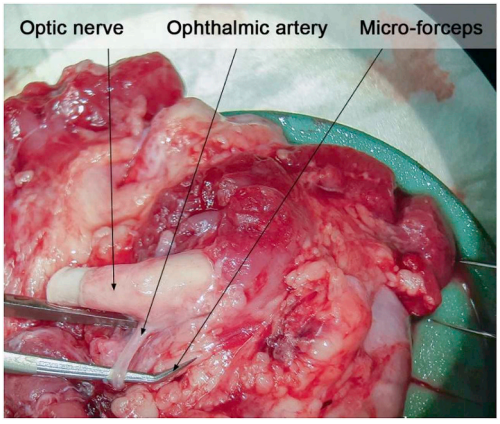

Fig. 4. Isolated ophthalmic artery cleaned by the surrounding connective tissue using micro-forceps.

euthanasia and were cannulated within 15 min (Abarca et al., 2013), while in the study of Gouras *et al.* feline eyes were cannulated 3–10 min after euthanasia (Gouras and Hoff, 1970). In two studies where bovine eyes were used (De Coo et al., 1993; Koeberle et al., 2006), the authors report blood vessel cannulation 1 h and 2 h after animal termination, respectively. However, the time window between animal termination and enucleation is not defined. In the present protocol, enucleation is recommended in animals whose carotid arteries were punctured for bleeding after death. Therefore, the blood is removed from the vascular network and formation of blood clots is limited. In a study where blood samples were obtained from human cadavers 1 h post mortem, fluid and coagulated blood was found regardless of the cause of death (Mole, 1948). Thus, if bleeding is not possible to be performed after animal death, the enucleation procedure should be completed as fast as possible after termination, but preferably not later than an hour.

It is useful to check all the enucleated eyes before starting the dissection process and discard those that are not in a proper condition. Some examples are: eyes whose optic nerve is too short, the optic nerve or proximal tissues are damaged, the meningeal sheath is detached from the optic nerve, the cornea is damaged. In addition, in the case that the isolation of the ophthalmic artery takes excessive amount of time, it is a good practice to switch to another eye that is in good condition. Based on our experience, approximately 60% of the enucleated eyes are in proper condition for cannulation, and 70% of the total amount of eyes in good condition is successfully cannulated by a trained person.

Another important point is the distinction of retinal artery from retinal vein, which run alongside the optic nerve. As a general rule, veins have thinner and more elastic walls, whereas arteries have thicker walls, therefore the remaining, native blood is less visible. In case the artery is isolated but its ending is detached from the optic nerve, make a knot to fix the free ending of the artery on the optic nerve.

If the preserved part of the ophthalmic artery is not long enough for cannulation and it is too proximal to the branching point of the short and long posterior ciliary arteries, and the central retinal artery, cannulation of the branch of the central retinal artery can be an alternative. However, it is recommended to avoid this because the ocular tissues perfused by the lacrimal artery (e.g. conjunctiva) and long and short posterior ciliary arteries (e.g. posterior uveal tract) will not be perfused (Fig. 3).

In case the perfusion is tested successfully without leakage in the cannulated area being observed, but nevertheless fluorescein is not visible in the retinal blood vessels, most likely a different vessel rather than the ophthalmic artery or the central retinal artery has been cannulated. Check if fluorescein is visible in other parts of the eye (e.g. if the sclera becomes colored). If that is the case, disconnect the cannula and search for the ophthalmic artery. If not, the presence of blood clot (s) in the ophthalmic artery might be blocking the perfusion. However, using the protocol described in Section 3, we have never experienced any perfusion issues due to blood coagulation in the ophthalmic artery.

This system can be further expanded or adapted depending on the intended objectives of the experimental study. Particularly in applications where longer perfusion periods are needed, heparinized porcine blood or physiological buffers enriched with nutrients (e.g. Krebs-Henseleit solution) can be used for perfusion. If gas mixtures are necessary (e.g. O_2, CO_2) then the syringe pump can be replaced by a peristaltic pump. The physiological solution can be mixed and saturated with gases prior to the cannulation point. Adjusting the temperature to physiological conditions can be achieved by pre-heating the tubing of the system using a heat exchanger just prior to the cannulation point. Additionally, the eye can be placed inside a container that is heated at 37 °C.

One limitation of the system described is that it only consists of an inlet (cannulation of the ophthalmic artery), with the venous drainage (outflow) being free. In this case the system is open. As a consequence,

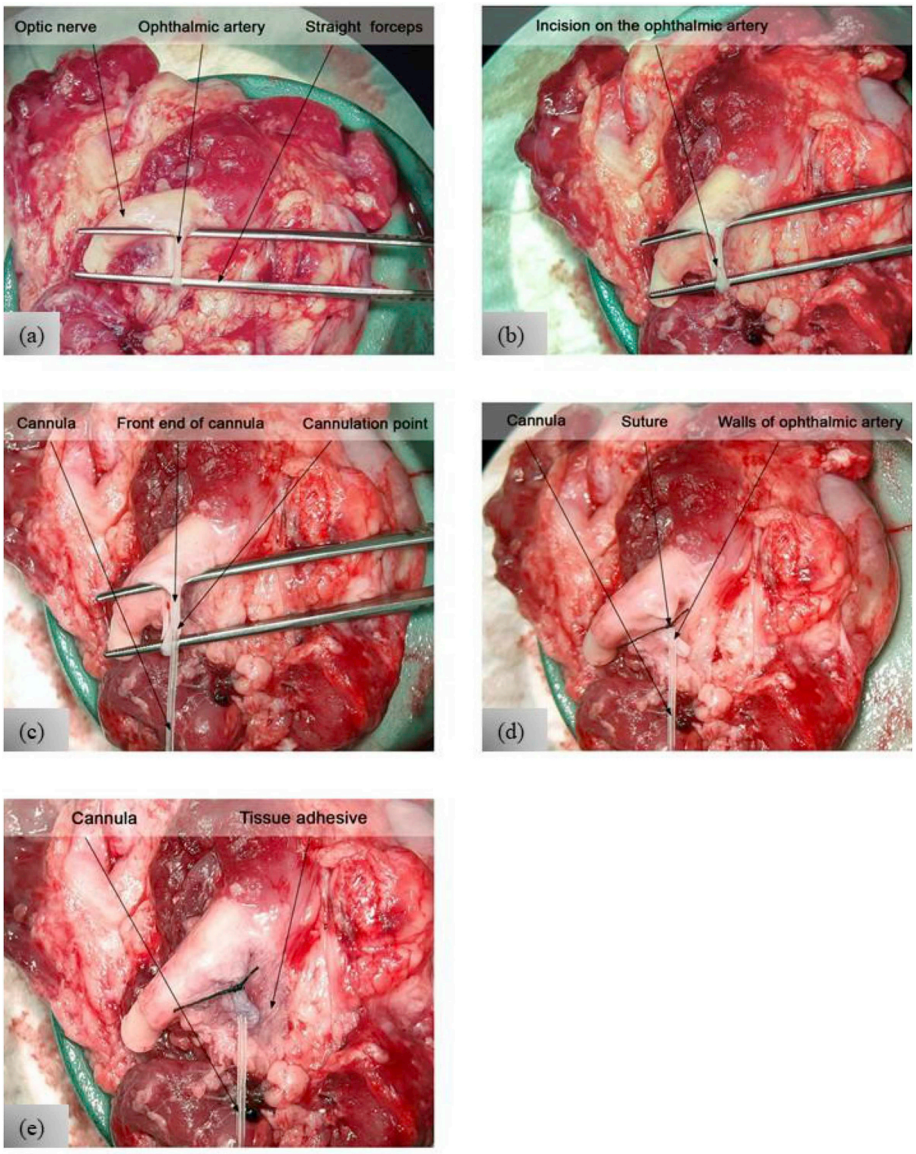

Fig. 5. Cannulation steps: the ophthalmic artery is kept stretched using straight forceps (a) and an incision is made using spring scissors (b). The cannula is subsequently inserted in the artery, with 0.5–1 cm of its front end surrounded by the artery walls (c). Suture is used to fix the cannula (d) and 3–4 tissue adhesive drops are added to ensure tight cannulation (e).

the perfused substances are not re-circulated. If a closed system is required (e.g. to evaluate the distribution of pharmaceutic compounds), the eye can be placed in a container in which all outflow fluids will be collected and analyzed. The syringe pump should then be replaced by a peristaltic pump, which will re-circulate the outflow fluids back to the inlet point.

The duration of tissue viability when physiological buffers are perfused can be evaluated over time with various methods, depending on the specific application. Some examples are: (i) activity of lactate dehydrogenase (LDH), (ii) measurement of the blood (or perfused

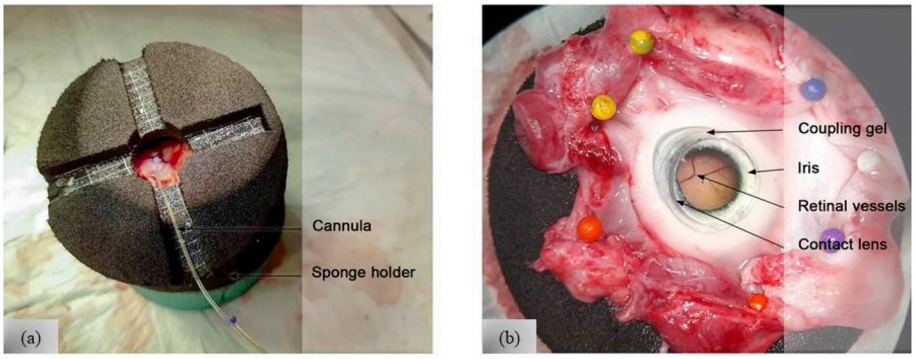

Fig. 6. The eye is placed inside the sponge holder, which is wetted with normal saline in order to keep the tissues in physiologic humid conditions (a). The cannula is fixated in the holder channel that is parallel to the ophthalmic artery. The holder is flipped with the anterior tissues of the eye facing upwards, and pins are used to fix the periocular tissues (b). The cornea is centrally aligned. Coupling gel is added on the surface of cornea and a contact lens is used to improve the field of view of the retina. The microscope focus is adjusted to have a clear image of the optic disc and the retinal blood vessels before the injection of the perfusion test solution.

solution) flow using Doppler ultrasonography, (iii) measurement of the intra-ocular pressure (IOP) and (iv) histological evaluation of tissue deterioration. If the system is used to test new surgery equipment, viability of tissues is required normally for shorter periods.

Synergistic or antagonistic interactions between heparine and antibiotics, phenothiazines and anti-inflammatory agents have been previously reported (Colburn, 1976). Therefore, if the system is utilized in pharmaceutical testing, any possible interactions between heparine and the compound under investigation should be examined in advance. No other heparin complications that could affect the functionality of the *ex vivo* system are known.

Hypothermic storage of eyes obtained post-mortem for keratoplasty purposes in humans requires storage of tissue in culture medium at 2–6 °C (Elisabeth et al., 2008). At these conditions tissue can be preserved for 7–10 days. Reinhard *et al.* investigated the post-mortem ganglion function using multielectrode arrays in enucleated minipig eyes (Reinhard et al., 2016). According to this study, ganglion activity was maintained for at least 50 h when the eyes were stored at ischemic and hypothermic conditions (4 °C). To the best of our knowledge, data describing the speed of the post-mortem deterioration of the posterior ocular segment and retinal detachment is lacking from the literature. The maximum time window between enucleation and preparation we tested was 6 h. During this period no evidence of tissue deterioration was observed. However, a viability study in order to measure the exact time frame for successful hypothermic preservation of porcine eyes in heparinized saline will be performed in the future.

5. Conclusion

In this report a detailed description of the method for enucleation and arterial perfusion of porcine eyes is given, supported by images and videos. This method can open new directions in the field of ophthalmology research by providing an alternative to *in vitro* and *in vivo* animal testing, using a cost- and time-effective protocol, and contributing to the reduction of laboratory animals in experimental research (the principles of the 3Rs).

Declaration of interest

No potential conflict of interest was reported by the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2019.02.003.

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