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TITLE:

Porcine Corneal Tissue Explant to Study the Efficacy of Herpes Simplex Virus-1 Antivirals

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KEYWORDS:

ex-vivo, cornea, herpes simplex virus type-1, antiviral, BX795, ocular infection

SUMMARY:

We describe the use of a porcine cornea to test the antiviral efficacy of experimental drugs.

ABSTRACT:

Viruses and bacteria can cause a variety of ocular surface defects and degeneration such as wounds and ulcers through corneal infection. With a seroprevalence that ranges from 60-90% worldwide, the Herpes Simplex Virus type-1 (HSV-1) commonly causes mucocutaneous lesions of the orofacial region which also manifest as lesions and infection-associated blindness. While current antiviral drugs are effective, emergence of resistance and persistence of toxic side-effects necessitates development of novel antivirals against this ubiquitous pathogen. Although in vitro assessment provides some functional data regarding an emerging antiviral, they do not demonstrate the complexity of ocular tissue in vivo. However, in vivo studies are expensive and require trained personnel, especially when working with viral agents. Hence ex vivo models are efficient yet inexpensive steps for antiviral testing. Here we discuss a protocol to study infection by HSV-1 using porcine corneas ex vivo and a method to treat them topically using existing and novel antiviral drugs. The methods detailed may be used to conduct similar experiments to study infections that resemble the HSV-1 pathogen.

INTRODUCTION:

People suffering from ocular infections often incur vision loss[1]. With a high seroprevalence worldwide, HSV infected individuals suffer from recurring eye infections which lead to corneal scarring, stromal keratitis and neovascularization[2-5]. HSV infections have also shown to cause less

frequently, a range of serious conditions among immunocompromised, untreated patients like encephalitis and systemic morbidity[6-8]. Drugs like Acyclovir (ACV) and its nucleoside analogs have shown consistent success in curbing HSV-1 infection and even control reactivation yet the prolonged use of these drugs is associated with renal failure, fetal abnormalities and failure to restrict the emergence of drug-resistance to evolving viral strains[9-13]. Complexities associated with HSV-1 ocular infections, have been previously studied in vitro using monolayers and 3D cultures of human corneal cells and in vivo using murine or rabbit ocular infections. While these in vitro models provide significant data on the cellular biological components of HSV-1 infections, they, however, fail to mimic the intricate complexity of corneal tissue and do little to illuminate the dendritic spread of the virus[14]. In contrast, although in vivo systems are more insightful in showing infection spread in corneas and immune activation responses during HSV-1 infection, they do come with the caveat that they require trained investigators and large facilities for animal care to overlook the experiments.

Here we use porcine corneas as an ex vivo model to examine the HSV-1 infection induced wound system. Both the potential pharmacology of certain drugs as well as the cell and molecular biology of the wound system caused by the infection can be studied through tissue explant cultures. This model may also be amended for the use for other viral and bacterial infections as well. In this study, porcine corneas were used to test the antiviral efficacy of a preclinical small molecule, BX795. The use of porcine corneas was preferred due to ease of access and cost effectiveness. Additionally, porcine corneal models are good models of human eyes with the corneas being easy to isolate, adequately sized for infection and visualization and robust to handle[15]. Porcine corneas are also comparable to the complexity of human corneal models in both trans corneal permeability and systemic absorption[15]. By using this model for the study, we were able to elucidate how BX795 is worthy of further investigation as a competent inhibitor of HSV-1 virus infection and adds to the literature of classifying it as a potential small-molecule antiviral compound[16].

PROTOCOL:

All the porcine tissue used in this study was provided by a third-party private organization and none of the animal handling was performed by University of Illinois at Chicago personnel.

1. Materials

1.1. Reagents

1.1.1. Use following reagents for Plaque assay: powder methylcellulose, Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin (P/S) for Plaque assay.

1.1.2. Use crystal violet tablets and ethanol (molecular biology grade) for preparing crystal violet solution for plaque assay.

1.2. Vero cell growth media – DMEM whole media

1.2.1. Open the DMEM media bottle inside the tissue culture hood. Remove 55 mL of media from the bottle using a serological pipette and discard it. Add 50 mL of FBS of P/S to DMEM. Refrigerate at 4 °C.

1.3. Plaque assay media – Stock of 5% methylcellulose media

1.3.1. Measure 2.5 g of methylcellulose powder with a stirring magnet inside a 500 mL glass bottle and autoclave it. After the bottle cools to room temperature, take 500 mL of DMEM whole media containing 50 mL of FBS and 5 mL of P/S and add it to glass bottle containing methylcellulose.

1.3.2. Stir this at 4 °C for 2 days using a magnetic stirrer. Refrigerate at 4 °C.

1.4. Media preparation for excised porcine cornea

1.4.1. Open a minimum essential media (MEM) bottle inside the tissue culture hood. Discard 10 mL of MEM from the bottle using a serological pipette.

1.4.2. Add 5 mL of insulin-transferrin-selenium (ITS) and 5 mL of antimycotic-antibiotic (AA) to the media and refrigerate at 4°C.

1.5. Master and working stock of crystal violet:

1.5.1. To make the crystal violet stock solution, add 1 g of crystal violet powder to 100 mL of 20% ethanol in water. This stock (1% w/v crystal violet) can be stored and used for up to year when stored in a place that is dark.

1.5.2. To make a working stock from this, add 50 mL of original stock solution to 350 mL of water. Add 100mL of ethanol to this solution to make a 500 mL working crystal violet solution (0.1% w/v crystal violet).

NOTE: Both these solutions need to be stored in the dark.

2. Procedure

2.1. Isolation of Porcine corneas from whole eyes[17]

2.1.1. Upon receiving the porcine eyes from a suitable vendor, store on ice if there is a delay with tissue processing as pictured in **Figure 1.**

2.1.2. Ensure that personal protection equipment is used and worn during this procedure to avoid contamination as well as accidents from spillage of vitreous humor.

2.1.3. Spray the working area with 70% ethanol to clean and disinfect. To ensure the working space is stable, spread a bench cover and tape down the sides securely as pictured in **Figure 2**.

2.1.4. Place porcine eyes on gauze (**Figure 3**). Using 50 mm gauze, hold posterior section (**Figure 4A**) of porcine eyes as shown in **Figure 4B** with one hand.

2.1.5. With a 30 G needle, gently make a single poke at approximately the center of epithelial surface of the eye carefully and ensure that there no damage to stroma (**Figure 5**). The poke should be limited to the epithelium (~100-200 µm) to avoid stromal involvement.

2.1.6. Using a sharp sterilized blade, make a small incision on the sclera at 1 mm distance from the cornea. Cut the edge of the cornea ensuring that the vitreous humor does not leak using a swift and smooth rotating action of the hand (**Figure 6A**). By holding the cornea at the cornea-sclera edge with a flat tweezer, cut off the remaining tethering membranes of eye using the blade (**Figure 6B**).

2.1.7. Take the cornea and place it in a 12-well plate overlaid with 2 mL of cornea medium. The cornea should be placed facing up, demonstrated in a series of steps in **Figures 7A-D, Figure 8**).

2.1.8. Add 5 µL of the virus solution containing 5 x 10^5 plaque forming units (PFU) of 17 GFP to the debrided site on the corneal surface. Place the 12-well plate containing infected porcine corneas in an incubator with 5% CO_2 for 72 h.

2.1.9. Spray any additional eyes not used in experiment with 10% bleach and securely disposed in biohazard bags.

2.2. Visualization

NOTE: The virus should be visualized every day prior to addition of drugs.

2.2.1. Turn on the stereo microscope and LED light source and allow lamp of machine to warm up before imaging the corneas. Carefully carry the plate of corneas to instrument without disturbing the solution. Change the filter so that GFP (380-460 nm) is used to look at specimens. Set the exposure time to 500 ms to capture images.

2.2.2. First, place the cornea plate under the stereoscope and capture the images at the lowest magnification of 7.5x. Follow this up with a series of increasing magnification images (e.g., 15x, 25x, 35x) such that all the viral spread and dendrite formation is visualized clearly

2.2.3. Make sure to return the infected corneas back into the tissue culture incubator and save all the images that were taken.

2.3. Virus infection quantification

NOTE: Virus titers from porcine corneas should be evaluated every day to analyze the effect of drug treatment.

2.3.1. To quantify virus, seed Vero cells at a density of 5 x 10^5 of cells per well if using a 6-well plate as done in this experiment. Do this a day prior to the infection. Incubate the plated cells overnight to ensure they are confluent for virus quantification.

2.3.2. Aliquot 500 µL of serum free media into multiple microcentrifuge tubes. Insert sterile cotton tipped swab dipped into the serum free media filled tubes. The cotton swabs need to be dipped and wetted for at least 5 min prior to the use.

2.3.3. Without disturbing the underlying media, transport the infected porcine cornea plate into a biosafety cabinet. Using the wet cotton swabs, make 3 revolutions clockwise and 3 revolutions anti-clockwise at a diameter of 5 mm from the center of the infected porcine cornea without applying excessive pressure.

2.3.4. Insert back the cotton swab into the serum free media filled microcentrifuge tube and rotate it clockwise and anti-clockwise 5 times. The metal tip of the cotton swab should be cut short so that it fits entirely into microcentrifuge tube and the lid can be closed.

2.3.5. Place the microcentrifuge tube containing the swabbed cotton tip on a vortex machine and vortex at high speed for 1 min.

2.3.6. Perform virus quantification via a plaque assay on these samples.

NOTE: This quantification step needs to be performed on days 2, 4 and optionally on 6 days post infection.

2.4. Virus quantification by Plaque assay

NOTE: To perform a plaque assay, grow and plate Vero cells into a 6 well plate and ensure 90% confluency of cells before start of assay. Use a confluent 75 cm^2 flask of these cells. All the steps below need to be performed inside a biosafety cabinet.

2.4.1. Wash the confluent monolayer of Vero cells in the flask with 10 mL of fresh phosphate buffer saline (PBS) after aspirating the culture medium. Repeat the wash step once again with PBS after aspirating the first set of wash solution.

2.4.2. Add 1 mL of 0.05% Trypsin to the cell monolayer. Incubate the flask at 37 °C for 5 min. With the naked eye, ensure that the cells are detached from the inside surface of the flask. If not, wait for another 5 min before examining the flask again. When cells appear to be detached, add

9 mL of whole media to the monolayer to ensure that the cells dislodge completely from the flask surface.

2.4.3. Collect all the cells from the flask along with the whole media and place them in a 15 mL centrifuge tube.

2.4.4. For every well of the 6 well plates that are used for plaque assay, use 300 µL from the centrifuge tube. Top up each well of the 6 well plate with 2 mL of whole media. Leave the plates in the incubator overnight to allow them to grow and form a confluent monolayer in each well.

2.4.5. In order to perform a plaque assay, perform a serial dilution of samples needs to be conducted before the quantification of virus.

2.4.6. Perform a $\log_{10}1$ fold dilution of the virus in micro-centrifuge tubes using serum free media until a dilution of 10^{-8} is reached. When at a 10^{-3} dilution, transfer 1 mL of the dilution to the monolayer of plated cells after aspirating the growth media on the cells from the 6 well plate, this will be the infection step. Incubate the infected plate at 37 °C incubator for 2 h.

2.4.7. Aspirate the existing infection media, wash with PBS twice gently to coat cells and add 2 mL of methylcellulose laden media per well to all 6 wells. Incubate for 72 h or until formation of plaques can be seen. Plaques can be identified by the formation of small gaps between cells in the cell monolayer.

2.4.8. Add 1 mL of methanol slowly to the corner of each well, using the wall as guiding tip. Incubate the 6 well plate at room temperature for 15 min. Slowly aspirate the contents of each well from the plate without disturbing or agitating the cell monolayer.

2.4.10 Add 1 mL of crystal violet working solution to each well of 6 well plate, ensuring all cells are covered. Incubate the 6 well plate in the dark for 30 min. Discard the crystal violet solution by aspirating it and dry the wells on a sheet of absorbent paper.

2.4.9. Count the number of plaques at the highest dilution well to quantify the total virus content in the starting solution. Repeat the process twice to confirm the viral titer.

REPRESENTATIVE RESULTS:

To understand the efficacy of the experimental antivirals, they need to be tested extensively before they are sent for in vivo human clinical trials. In this regard, positive control, negative control and test groups have to be identified. Trifluorothymidine (TFT) has long been used as the preferred treatment to treat herpes keratitis topically[16]. Used as a positive control, the TFT treated corneal groups show lower infection spread. As a negative control, we used DMSO or vehicle control dissolved in PBS. BX795, the experimental preclinical drug was the test group. A total of 4 corneas were assigned to each group and the drugs were added 3-times every day to the porcine corneas. Our results using stereoscopic fluorescence imaging show that the antiviral efficacy of BX795 is similar to TFT in controlling viral spread. Viral spread in our studies can be

visualized by imaging the green fluorescence channel in the stereoscope. We observed that vehicle only treated negative control group corneas showed spread of the virus from the central infection zone to its periphery by 6 days post initial viral inoculation, while both drugs (BX795 and TFT) clear the infection in day 4 - 6 images (**Figure 9A**). Similarly, the ocular swabs taken on days 2 and 4 post infection show a complete inhibition of virus in the positive control and BX795 treated samples while a sharp increase in infectious virus titer is observed in the negative control group (**Figure 9B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Porcine eyes kept on ice until tissue is processed.

Figure 2: Work bench setup.

Figure 3: Porcine eyes placed on gauze.

Figure 4: Porcine eye with 30G needle pictured.

Figure 5: 30 G needle used; hole made at center of epithelial surface.

Figure 6: Rotating action used to cut around corneal edge using sharp sterilized blade (A,B).

Figure 7: Cornea images. (**A,B**) The cornea finally cut using sharp scissors (**C**) Isolated cornea is held by tweezers (**D**) Completely isolated cornea, ready for use

Figure 8: Corneas placed in 12-well plate and incubated for 72 h.

Figure 9: Progression of viral spread taken during course of infection. Freshly excised porcine corneas were infected with HSV-1 17-GFP and (**A**) imaged using microscope on days 2, 4 and 6 post infection. Topical treatment with DMSO, TFT or BX795 was started on day 2 post infection. (**B**) Plaque assays were performed using swabs taken from the porcine corneas on Vero Cells. Two-way ANOVA test was performed to understand significant differences between the treatment groups. n=4, ****p=0.0001.

DISCUSSION:

Prior research has shown BX795 to have a promising role as an antiviral agent against HSV-1 infection; by inhibiting the TANK-binding kinase 1 (TBK1)[16]. Both TBK1 and autophagy have played a role in helping inhibit HSV-1 infection as demonstrated on human corneal epithelial cells. BX795 was shown to be maximally effective with antiviral activity at a concentration of 10μM and using both western blot analysis and viral plaque analysis of key viral proteins, BX795 was shown to inhibit HSV-1 infection comparable to the activity of TFT[16]. The study on porcine corneas followed the same analysis and obtained similar results as demonstrated above; BX795 is shown to be just as effective as TFT in inhibiting infection – images taken of porcine corneas at day 4 and 6 of infection show comparable results in both TFT and BX795. Plaque assays performed to quantify

secreted virions also reinforce these findings[16]. BX795 has also shown to have antiviral effects in vitro and its use topically in mouse models in vivo has also shown suppression of corneal HSV-1 infection[16].

The study contributes to establishing BX795 as an effective and leading compound for broad-spectrum antiviral application against HSV-1 infection. By showing its efficacy in porcine models as comparable to TFT, BX795 stands to be successful in multiple infection models[16]. Additionally, BX795 is important as it has been shown to be effective in multiple virus strains, even HSV-1 (KOS)tk12 which is resistant to another widely used drug Acyclovir (ACV)[16]. BX975 treated cells show very little expression of HSV-1 viral protein gB which certify its inhibition efficacy of HSV-1 virus. BX795 is also demonstrates better anti-viral efficacy at lower doses compared to other treatments and anti-herpesvirus therapies. Furthermore, therapeutic concentrations of BX795 (even proposed concentration of 10 µM) show no adverse cytotoxicity towards cells – no apoptosis inducement and cell death, which is again comparable to the TFT control[16].

Critical steps within the protocol section include isolation of porcine cornea from the whole eye. This involves the debridement of the cornea at the center of the eye using a needle and requires very little force to ensure no stromal involvement followed by excising the cornea from the ocular surface gently without disturbing the iris. Another set of critical steps include parts involving pre-wetting cotton tips and swabbing the corneal surface. The motion should be gentle to ensure corneal epithelium is not being dislodged from the ocular surface.

Modification can be done to the epithelial debridement step. Instead of using a 30 G needle to make a single poke at the center of the cornea, the experimenter can use a sterile blade or 30 G needle to gently make grid shaped scratches to the corneal epithelium. This ensures robust infection to the epithelium.

Porcine corneas should be used on the same day of procurement and should not be held for longer than 24 h. Porcine corneas kept in ice for longer than 4 h will result in the iris sticking to cornea. This makes it harder to separate the cornea from the rest of the eye during excision. Not all porcine corneas will get infected alike. The experimenter should infect a minimum of 5 eyes per group and then pick equally infected corneas on day 2 post infection to proceed with the experiment.

The significance of the current technique involves the cost effectiveness of using porcine over human corneas. The technique is also significant because of the relative freshness of the corneas being used when compared to human corneas.

Future applications of the current technique include but not limited to its use in testing drug permeability studies, ex vivo pharmacokinetic and pharmacodynamic studies. Porcine corneas can also be used to test antibacterial and antifungal drugs in addition to antiviral drugs. The corneas can also be used for ex vivo wound healing assays to study diabetic wounds.

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DISCLOSURES:

The authors declare no conflict of interest and no competing financial interests.

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Figure 1

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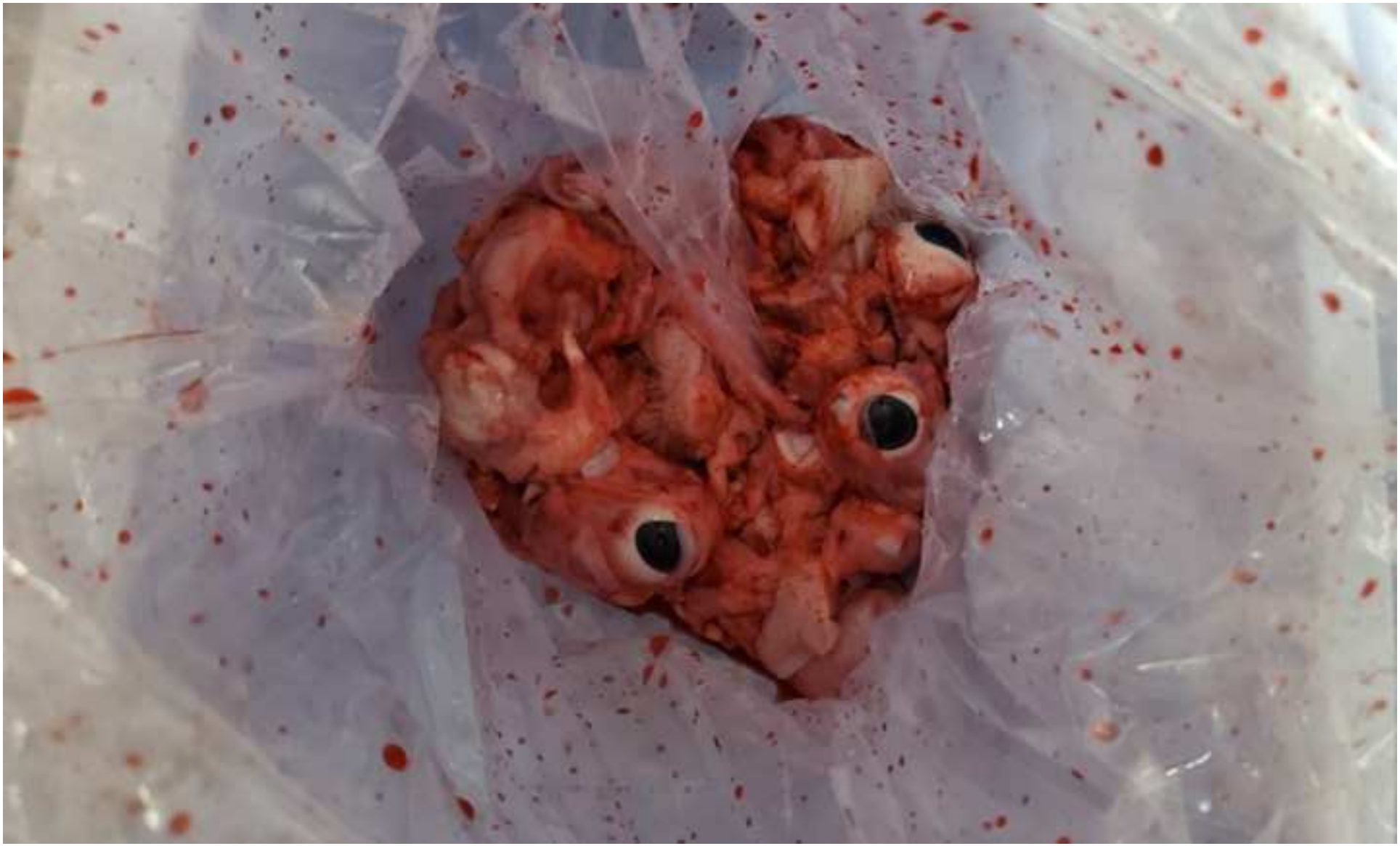

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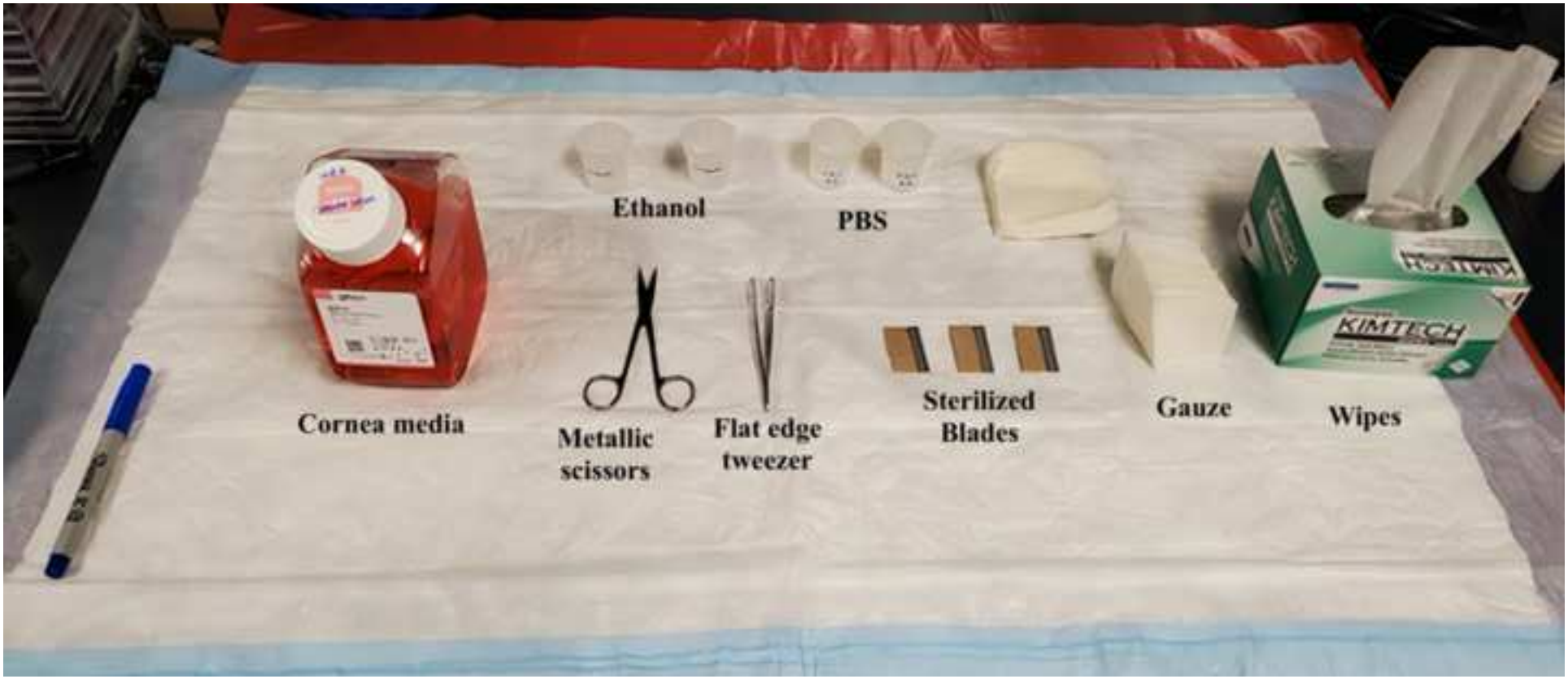

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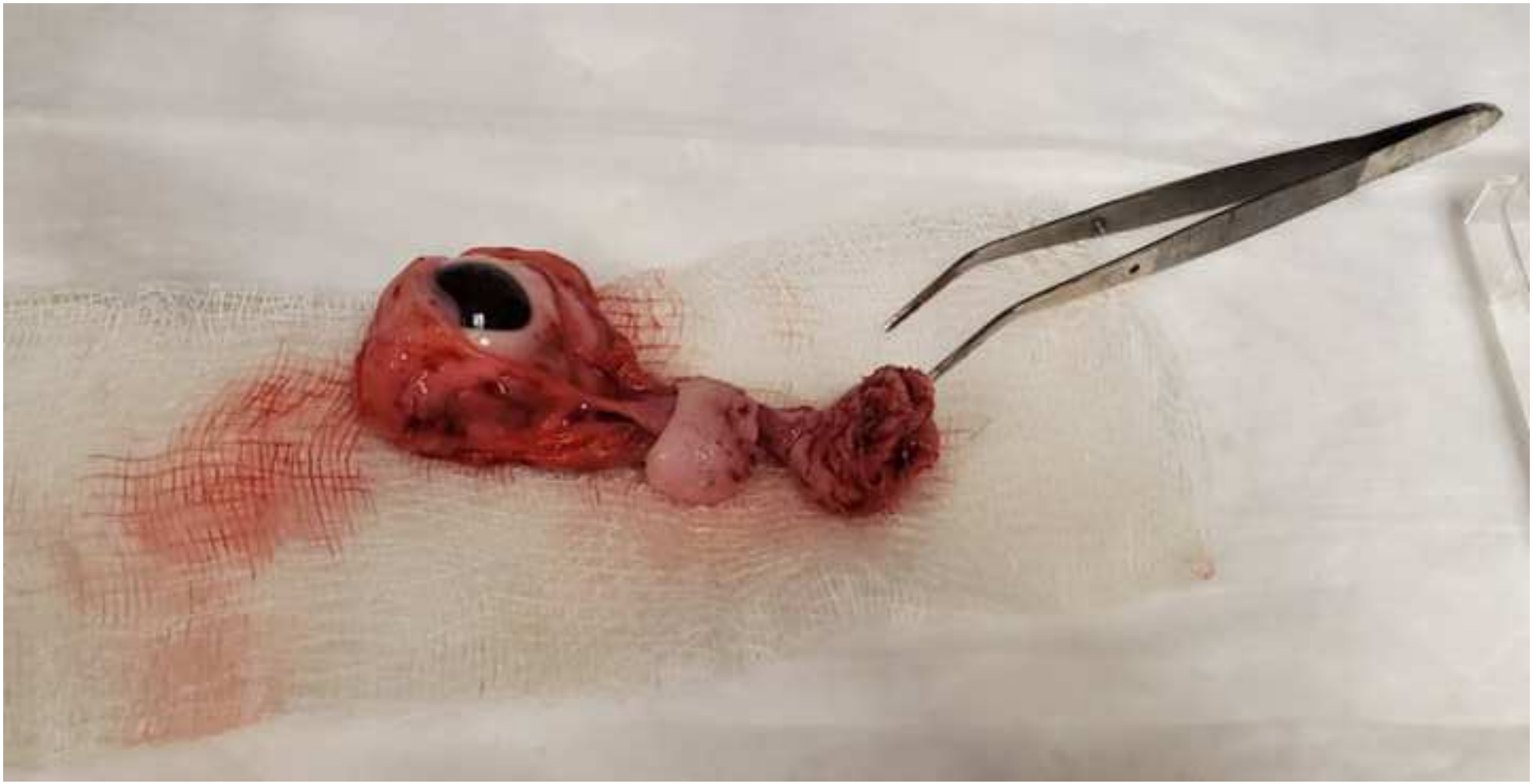

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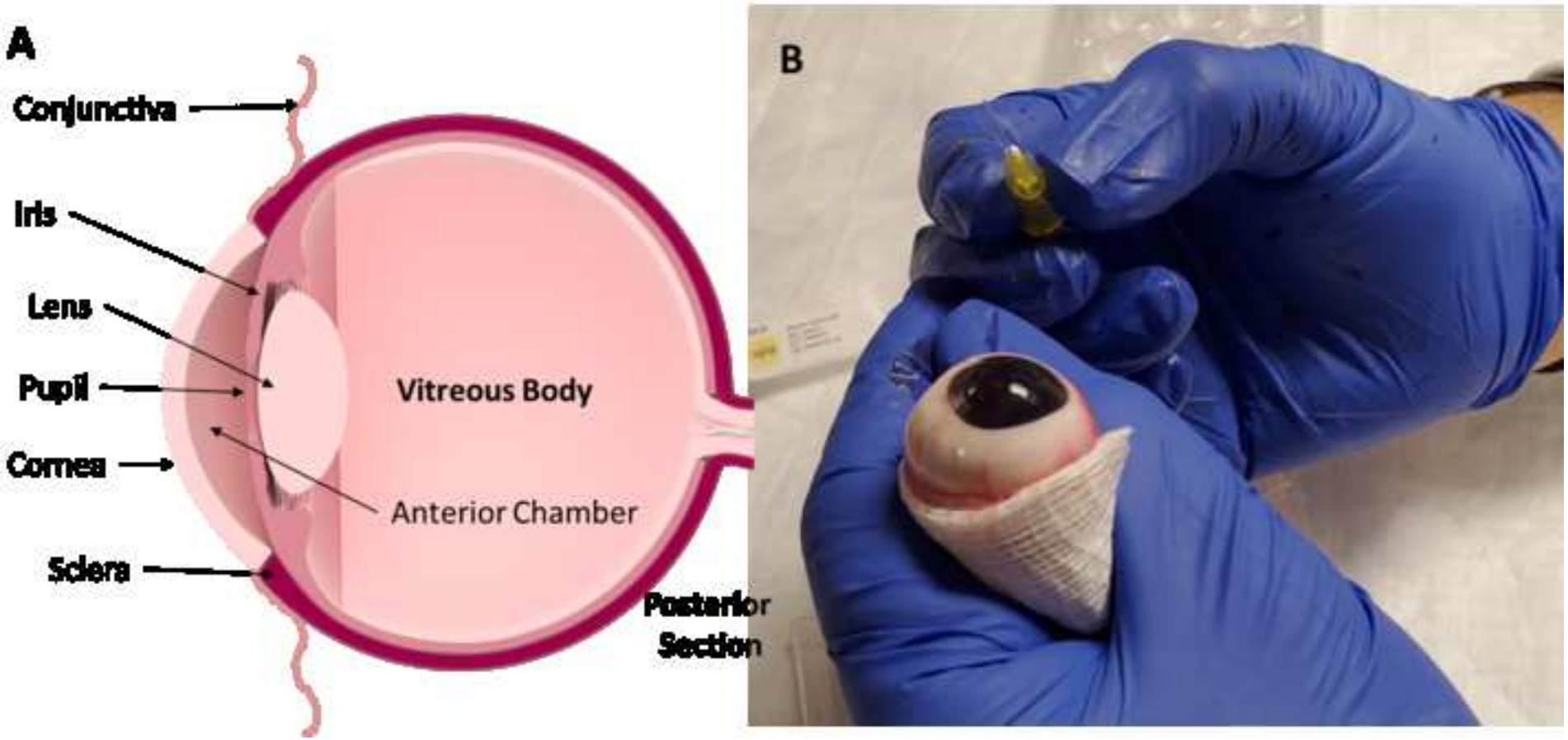

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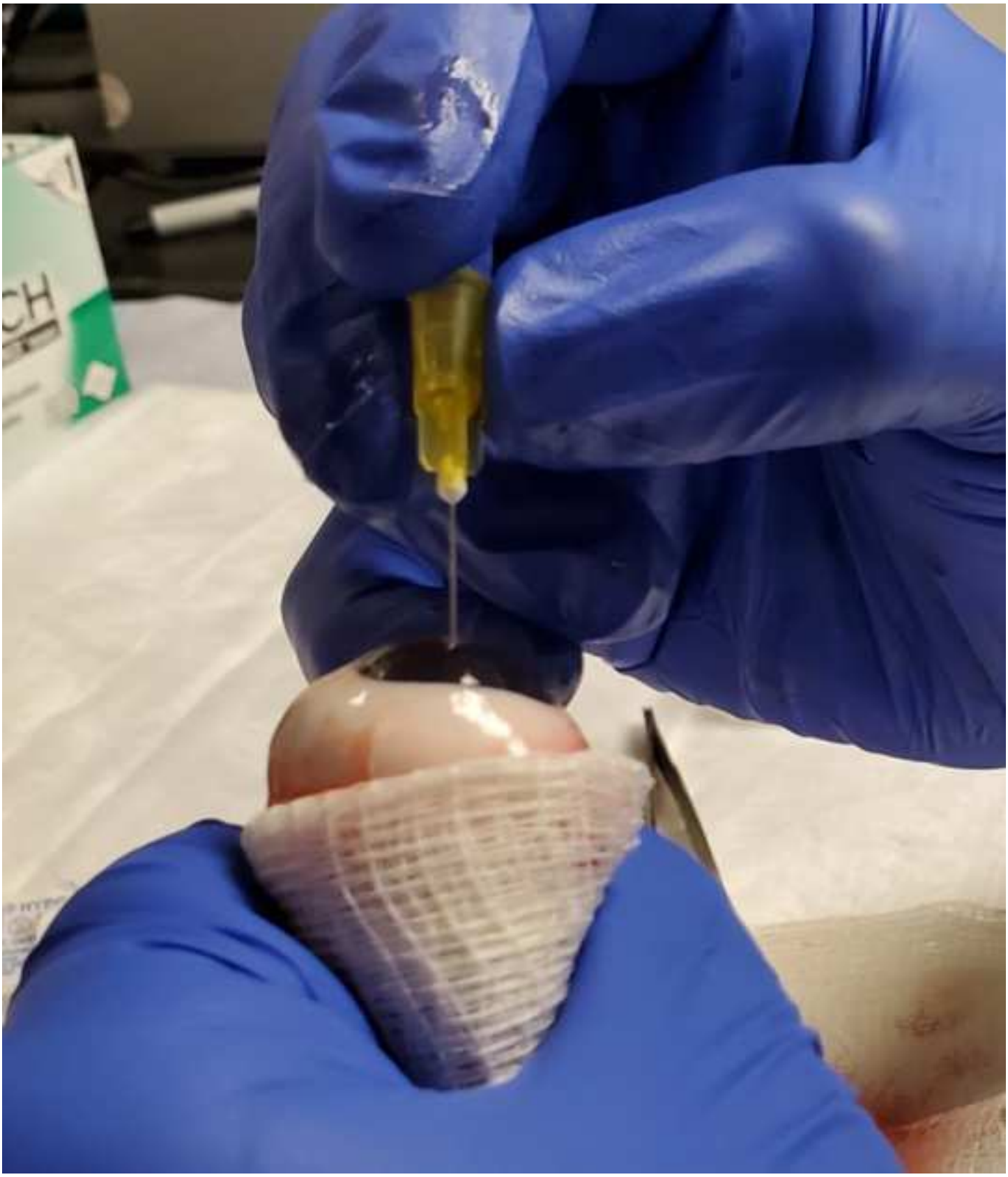

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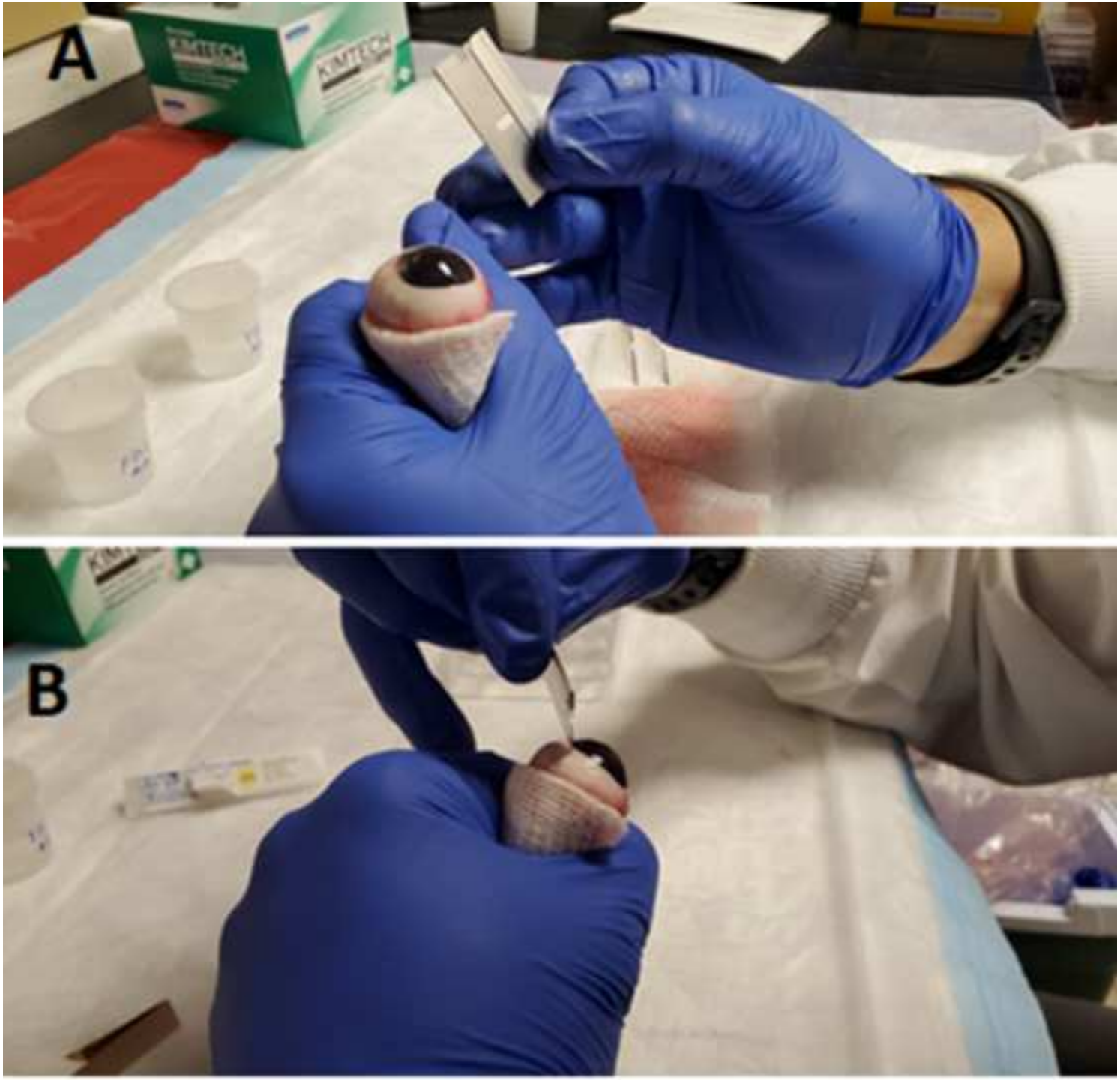

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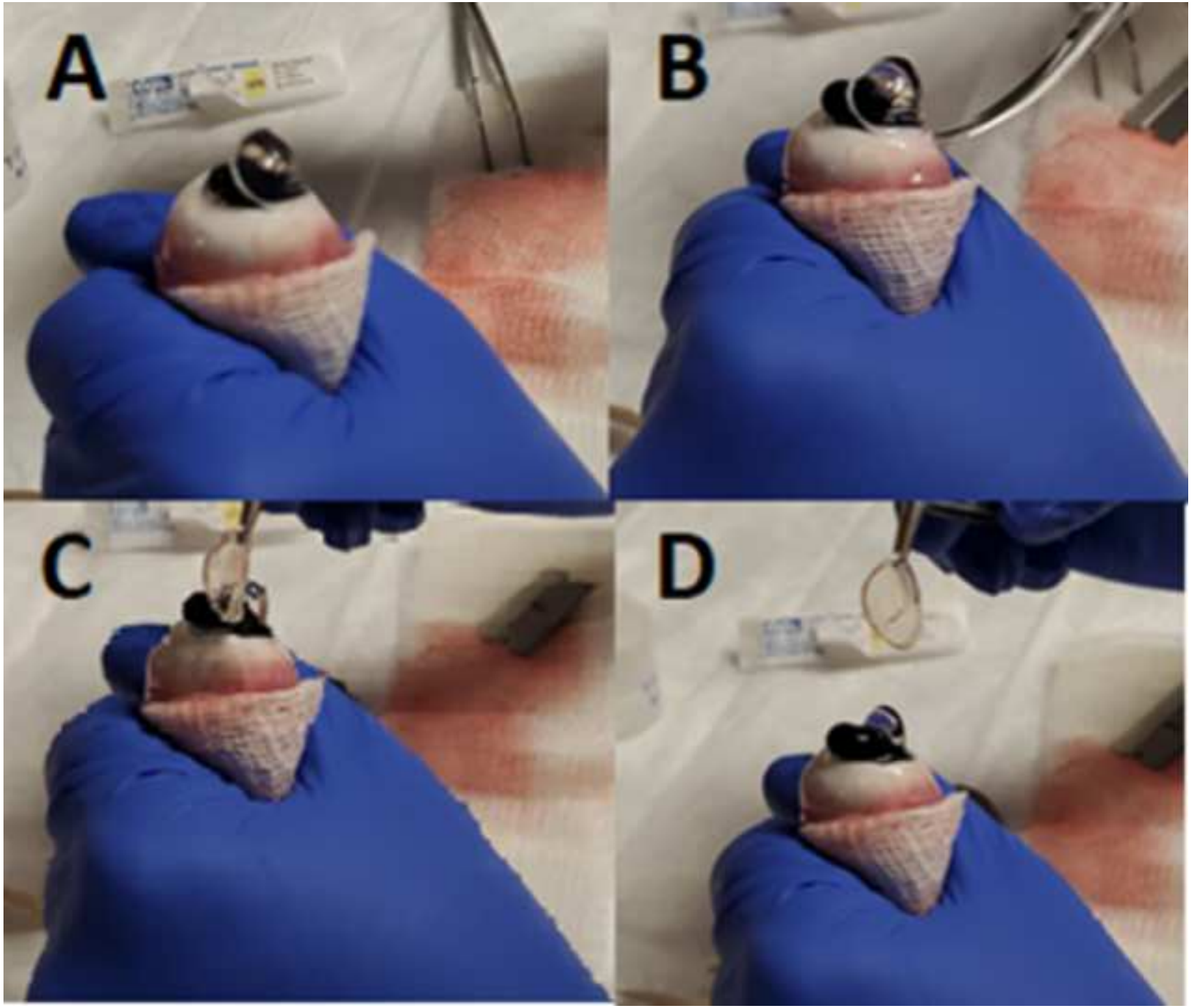

Figure 8

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Figure 9

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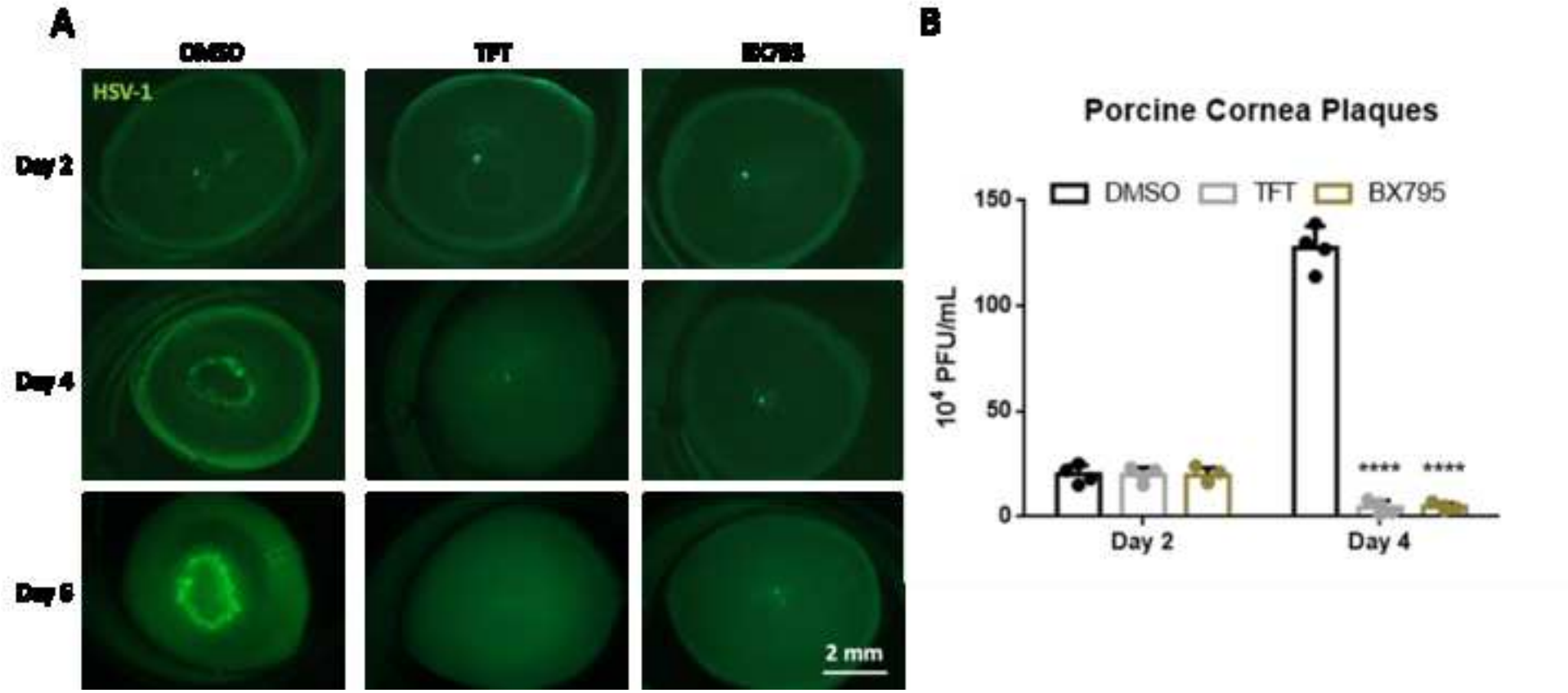

Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_62195.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
30 G hypodermic needles.	BD	305128	
500 mL glass bottle.	Thomas Scientific	844027	
Antimycotic and Antibiotic (AA)	GIBCO	15240096	Aliquot into 5 mL tubes and keep froz
Benchtop vortexer.	BioDot	BDVM-3200	
Biosafety cabinet with a Bio-Safety Level-2 (BSL-2) certification.	Thermofisher Scientific	Herasafe 2030i	
Calgiswab 6" Sterile Calcium Alginate Standard Swabs.	Puritan	22029501	
Cell scraper - 25 cm	Biologix BE	70-1180 70-1250	
Crystal violet	Sigma Aldrich	C6158	Store the powder in a dark place
Dulbecco's modified Eagle's medium - DMEM	GIBCO	41966029	Store at 4 °C until use
Ethanol	Sigma Aldrich	E7023	
Fetal bovine serum -FBS	Sigma Aldrich	F2442	Aliquot into 50 mL tubes and keep fro
Flat edged tweezers – 2.	Harward Instruments	72-8595	
Freezers --80 °C. -	Thermofisher Scientific	13 100 790	
Fresh box of blades.	Thomas Scientific	TE05091	
Guaze	Johnson & Johnson		108 square inch folder 12 ply
HSV-1 17GFP	grown in house	-	Original strain from Dr. Patricia Spears
Insulin, Transferrin, Selenium - ITS	GIBCO	41400045	Aliquot into 5 mL tubes and keep froz
Magnetic stirrer.	Thomas Scientific	H3710-HS	
Metallic Scissors.	Harward Instruments	72-8400	
Micropipettes 1 to 1000 µL.	Thomas Scientific	1159M37	
Minimum Essential Medium - MEM	GIBCO	11095080	Store at 4 °C until use
OptiMEM	GIBCO	31985047	Store at 4 °C until use

Penicillin/streptomycin.	GIBCO	15140148	Aliquot into 5 mL tubes and keep froz
Phosphate Buffer Saline -PBS	GIBCO	10010072	Store at room temperature
Porcine Corneas	Park Packaging Co., Chicago, IL	0	Special order by request
Procedure bench covers - as needed.	Thermofisher Scientific	S42400	
Serological Pipettes	Thomas Scientific	P7132, P7127, P7128, P7129, P7137	
Serological Pipetting equipment.	Thomas Scientific	Ezpette Pro	
Stereoscope	Carl Zeiss	SteREO Discovery V20	
Stirring magnet.	Thomas Scientific	F37120	
Tissue culture flasks, T175 cm2.	Thomas Scientific	T1275	
Tissue culture incubators which can maintain 5% CO2 and 37 °C temperature.	Thermofisher Scientific	Forma 50145523	
Tissue culture treated plates (6-well).	Thomas Scientific	T1006	
Trypsin-EDTA (0.05%), phenol red	GIBCO	25-300-062	Aliquot into 10 mL tubes and keep fro
Vero cells	American Type Culture Collection ATCC	CRL-1586	

en until use

zen until use

s, Northwestern University. GFP expressing HSV-1 strain 17
en until use

en until use

zen until use

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To
The Editor
JoVE

Dear Editor,

Thank you very much for the timely review of our article. The reviewers were very positive about our manuscript. In the revised manuscript, we have addressed all of the editorial and reviewer concerns. We have also prepared a line by line reply to Reviewer's comments and attached to this letter. We hope that you will now find that our manuscript suitable for publication.

Thank you very much for your attention to this submission. We look forward to hearing back from you soon.

Yours Sincerely,

Deepak Shukla, PhD

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Ans: We have gone through the manuscript again to ensure no grammatical errors exist.

2. Please provide an email address for each author.

Ans: Email addresses for each author have been provided

3. Please revise the following lines to avoid overlap with previously published work: 60-66, 170-172, 198-207, 215-218

Ans: The suggested lines have been revised to avoid overlap

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Ans: The references have been changed to superscripts

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Park Packaging Co., Chicago, IL; Gibco #XYZ; Sigma Aldrich # XYZ; ITS, Gibco, Life Technologies; SteREO Discovery V20 (Carl Zeiss Germany, Jena, Germany); Puritan Calgiswab; OptiMEM etc

Ans: All of the commercial language has been removed from the manuscript as per the suggestion.

6. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

Ans: An Ethics statement has been provided as follows

Ethics Statement:

All the porcine tissue used in this study was provided by a third party private organization and none of the animal handling was performed by University of Illinois at Chicago personnel.

7. Please move the information about materials in the protocol to the Table of Materials, which should contain information about essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Ans: We have tried to improve the protocol explanation to a greater extent to help the reader better understand the methods being explained in this manuscript. For section 2.2, the computer software being used is very subjective and hence we believe the button clicks on this software should be avoided to reduce confusion to the reader.

9. Line 213: How is virus quantification to be performed? Where is the Titration/Quantification section?

Ans: We have added a virus quantification section to the protocol section

10. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

Ans: All the requested changes have been made

11. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. The legends should appear only in the Figure and Table Legends section after the Representative Results.

Ans: All the embedded figures have been removed and uploaded separately.

12. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Ans: All figures taken with a microscope have now been labelled with a scale bar and the scale has been defined in the figure legend

13. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Ans: All the points mentioned have been addressed

14. After Acknowledgments, please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

Ans: A disclosure section has been added at the end of the manuscript.

15. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references. Do not abbreviate journal names.

<u>Reviewers' comments:</u>

Reviewer #1:

1. Is the methylcellulose autoclaved in powder form and then media added? If so , this should be directly stated.

Ans: Methylcellulose was autoclaved in powder form prior to adding whole media. This is now indicated in the manuscript in line 116.

2. Figure 1 should include arrows pointing to eyes.

Ans: We thank the reviewer for this suggestion but, given that all the tissues seen in the plastic bag are eyes, we think it might not be suitable to show arrows in the figure.

3. How deep is "poke" in fig 6 and what is the purpose of the poke.

Ans: The poke is intended to be approximately 100-200 micrometers deep to ensure no stromal involvement.

4. A figure showing labelled parts of the eye before naming them in the procedure would be helpful.

Ans: An additional eye anatomy picture has been added to figure 4 as per the suggestion of the reviewer.

5. What is the procedure for creating the 3x3 epithelial debridement grid on the corneal surface? This should be added.

Ans: This part has been removed from the current manuscript as it is confusing.

6. "….without causing any damage to the cornea". Does this mean that contact between the swab and the cornea is not made? How would one determine whether they were successfully swabbing without damaging? Is uniform swabbing important for comparing titers?

Ans: The wording has been changed to “Using the wet cotton swabs, make 3 revolutions clockwise and 3 revolutions anti-clockwise at a diameter of 5 mm from the center of the infected porcine cornea without applying excessive pressure”. Contact between the cornea and the swab is made. Excess pressure can remove the epithelium from the corneal surface. This should be avoided. This has been addressed in the manuscript

7. Additional brief discussion about controls and replicates would be helpful. Additional brief discussion about evaluating the quality of the corneas, and what issues can arise? For example how prevalent is contamination? Are the corneas inspected microscopically prior to focal debridement and infection to confirm that they are uniformly undamaged.

Ans: A section at the end of the manuscript explaining the critical steps, limitations and advanatages of using porcine cornea has been added for the reader’s benefit. Contamination is prevalent in the corneas if anti-fungal/anti-bacterial agent is not used. As we use this anti-anti mixture in our cornea media, we do not see much contamination in the porcine corneas.

Reviewer #2:

Minor Concerns:

1. In the introduction section, please briefly discuss why the pig eye was chosen, ie, what makes the pig cornea more suitable than mouse or guinea pig cornea. How does the infection course in pig cornea compare to other model systems? Does the virus replicate similarly in pig eye as in human/mouse eye?

Ans: We thank the reviewer for their valuable suggestion. A small segment which talks about the advantages of using porcine corneas has been added to the introduction at line 59-66. “The use of porcine corneas was preferred due to ease of access and cost effectiveness. Additionally, porcine corneal models are good models of human eyes with the corneas being easy to isolate, adequately sized for infection and visualization and robust to handle. Porcine corneas are also comparable to the complexity of human corneal models in both trans corneal permeability and systemic absorption. By using this model for the study, we were able to elucidate how BX795 is worthy of further investigation as a competent inhibitor of HSV-1 virus infection and adds to the literature of classifying it as a potential small-molecule antiviral compound.”

2. Where can readers obtain the GFP-expressing HSV-1 virus?

Ans: The readers can contact the corresponding author for obtaining the GFP-expressing virus.

3. Line 112: amount of P/S missing.

Ans: We thank the reviewer for pointing this out to us. 5 mL of P/S was added to the media. This correction has now been added to the manuscript.

4. Line 114: unclear- is the 2.5g methyl cellulose dissolved in liquid before autoclaving? How much? Is the final concentration really 5% methyl cellulose?

Ans: The line has been changed to make more sense. It currently reads as follows "Measure 2.5g of methylcellulose powder with a stirring magnet inside a 500 mL glass bottle and autoclave it. After the bottle cools to room temperature, take 445 mL of DMEM media, 50 mL FBS, 5 mL P/S and add it to glass bottle containing methylcellulose."

5. Line 122: what are the final concentrations of crystal violet and ethanol in the master and working stock solutions?

Ans: The stock is at 1% w/v of crystal violet and the working solution is at 0.1% w/v of crystal violet. These details have been added to the manuscript in the same section.

6. Line 142: Gauze is not mentioned in materials section.

Ans: All materials used are now mentioned in a separate excel sheet according to the editorial request.

7. Line 151: what length is the needle? Approximately how deeply should the needle be inserted?

Ans: The length of the needle is half inch. The depth to which the needle should be inserted is 100-200 micrometers to ensure no stromal involvement. However this level of precision is not usually possible and hence the needle needs to be gently inserted into the cornea. This aspect of the protocol has now been changed in the manuscript as follows "With a 30G needle, gently make a single poke at approximately at center of epithelial surface of eye carefully to ensure no damage to stroma (figure 5). The poke should be limited to the epithelium (~100-200 μm) to avoid stromal involvement."

8. Line 170: unclear what is the 3x3 epithelial debridement grid. Is the initial virus inoculum removed at any point?

Ans: This line has been removed from the manuscript to reduce confusion.

9. Materials list includes heat block and sonicator, but it's unclear what these are used for.

Ans: Thank you for pointing this out. These materials have been removed from the list