RESEARCH ARTICLE

REVISED Impact of freeze-thaw cytoablation on aqueous outflow patterns in *ex vivo* anterior chamber perfusion cultures and whole eyes [version 2; peer review: 2 approved with reservations]

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Abstract

Background: Porcine eyes have been widely used as *ex vivo* models in glaucoma research, as they share similar features with human eyes. Freeze-thawing is a non-invasive technique that has been used to obliterate living cells in anterior segment *ex vivo* cultures, to prepare them for further research such as cellular repopulation. This technique has previously been shown to reduce the intraocular pressure (IOP) in porcine eyes. The aim of this study was to investigate whether freeze-thaw cytoablation causes corresponding canalogram outflow changes in perfused anterior segment cultures (A_{FT}) and whole porcine eyes (W_{FT}). We hypothesized that the known IOP drop in A_{FT} after trabecular meshwork ablation by freeze-thaw would be accompanied by a similarly large change in the distal outflow pattern.

Methods: Two-dye (fluorescein and Texas red) reperfusion canalograms were used to compare the outflow time before and after two -80°C cycles of freeze-thaw. We assigned 28 freshly enucleated porcine eyes to four groups: perfused anterior segment dye controls (A_{CO} , $n = 6$), perfused whole eye dye controls (W_{CO} , $n = 6$), freeze-thaw treated anterior segment cultures (A_{FT} , $n = 10$), and freeze-thaw treated whole eyes (W_{FT} , $n = 6$).

Results: In control groups A_{CO} and W_{CO} , the two different dyes had similar filling times. In A_{FT} , the outflow pattern and filling times were unchanged. In W_{FT} , the temporal superior quadrant filled more slowly ($p = 0.042$) while all others remained unchanged. The qualitative appearance of distal outflow spaces was altered only in some eyes.

Conclusions: Freeze-thaw cytoablation caused neither loss nor leakage of distal outflow structures. Surprisingly, the loss of an intact

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trabecular meshwork over the entire circumference did not result in a general acceleration of quadrant outflow times. The results validate freeze-thawing as a method to generate an extracellular matrix without major structural changes.

Keywords

freeze-thaw, porcine eyes, trabecular meshwork, canalography

This article is included in the **Eye Health** gateway.

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REVISED Amendments from Version 1

The article has been adjusted to meet reviewer comments. Changes have been made to the introduction and discussion. In our introduction we now clarify the reason for choosing this technique for decellularization. Furthermore, we now discuss the potential washout of cellular debris post-ablation.

Any further responses from the reviewers can be found at the end of the article

Introduction

The trabecular meshwork represents a location of great interest for glaucoma research, and non-invasive methods are needed to decellularize it and convert it into a scaffold for cellular transplantation. As porcine eyes share several important features with human eyes¹⁻³, can be used to mimic surgical interventions^{4,5} and different glaucoma types, including secondary open angle⁶ and angle closure glaucomas⁷, they have been widely used as *ex vivo* models in glaucoma research. In contrast to human donor eyes, they are also ubiquitously available and remain responsive to stimuli and drugs when freshly harvested. This allows the exploration of anatomical functions^{3,8} and the effects of biologicals⁹, drugs¹⁰, and surgical interventions¹¹. Corresponding outflow changes can be studied with high spatial and temporal resolution^{5,12,13}.

Ab interno trabeculectomy of the nasal circumference increases outflow in whole porcine eyes as well as anterior segment cultures⁵. Outflow is changed mostly at the site of ablation but also enhanced circumferentially⁵. In contrast to *ab interno* trabeculectomy, freeze-thaw decellularization is a nonspecific and site-agnostic method to remove all living cells from eye cultures to ready them for the transplantation and study of cells of interest^{14,15}. In perfused porcine anterior segments, this is accompanied by a significant reduction in intraocular pressure (IOP)¹⁶. Freeze-thawed scaffolds may be easier to generate than artificial three-dimensional trabecular meshwork structures¹⁷⁻¹⁹.

Here, we hypothesized that subjecting porcine eyes to freeze-thaw decellularization would result in faster outflow throughout the entire circumference and alter the outflow pattern due to a loss of endothelial integrity of the distal outflow tract. We opted to use this cytoablation technique as we have shown in previous studies that it provides a complete ablation of the trabecular meshwork effectively while keeping its cytoskeletal structure intact¹⁶.

Methods

Study design

Twenty-eight porcine eyes were assigned to one of four groups: anterior segment control (A_{CO} , $n = 6$), and whole eyes control (W_{CO} , $n = 6$), freeze-thaw anterior segment (A_{FT} , $n = 10$), freeze-thaw whole eyes (W_{FT} , $n = 6$). Eyes in the experimental group (A_{FT} and W_{FT}) underwent two cycles of freeze-thaw at -80°C . Reperfusion canalograms were carried out in the

two control groups that did not undergo freeze-thaw (A_{CO} and W_{CO}), as well as before and after freeze-thawing in the experimental groups (A_{FT} and W_{FT}). No live vertebrate animals were used in this study. Pig eyes were obtained from a local abattoir. As a result, no ethics approval was required.

Preparation of porcine anterior segments and perfusion system

Freshly enucleated porcine eyes were obtained from a local abattoir (Landschlachtereier Issing, Retzbach, Bavaria, Germany) and processed within three hours of death. All eyes were placed in a 5% povidone-iodine solution for three minutes, and rinsed twice with phosphate-buffered saline (PBS) (Dulbecco's Phosphate Buffered Saline, Sigma-Aldrich, St. Louis, Missouri, USA). The laterality (left versus right eyes) was determined by examining the extraocular muscles and the shape of the cornea. The extraocular tissues were removed for all eyes. A_{FT} and A_{CO} eyes were then bisected at the equator. This was followed by the removal of the vitreous body, lens, and uvea. The eyes were then rinsed with PBS, mounted on perfusion dishes and infused with Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Life Technologies, Carlsbad, California, USA) at a constant pressure of 15 mmHg using a 20 ml syringe at a height of 20 cm above the perfusion chamber.

Whole eyes were mounted facing up using a specimen vial (Wheaton CryoElite Tissue Vial #W985100, Wheaton Science Products, New Jersey, USA). A 30G needle was subsequently inserted bevel-up into the anterior chamber through the temporal cornea. Perfusion was maintained for 20 minutes in both anterior segments and whole eyes.

Canalograms and freeze-thaw cycles

Perfusion with fluorescein: After 10 minutes of perfusion with DMEM, an anterior chamber exchange was performed in A_{FT} and A_{CO} by opening the outflow and allowing the anterior chamber to empty before the infusion was switched to 0.017 mg/ml fluorescein (fluorescein solution 10%, Alcon, Freiburg, Switzerland). The outflow port was then closed and the canalogram images were captured at 30-second intervals. In whole eyes, a safe anterior chamber exchange could not be performed without injuring the lens capsule and was therefore omitted.

Freeze-thaw cycles: The treatment groups A_{FT} and W_{FT} underwent two cycles of freeze-thaw. These consisted of freezing at -80°C for two hours, followed by thawing at room temperature for one hour¹⁶.

Perfusion with Texas red: Another full anterior chamber fluid exchange was performed in anterior segments after the freeze-thaw cycles by connecting a 20 ml reservoir containing 0.28 mg/ml Texas red (Sulforhodamine 101, 25 mg crystalline solid, Hycultec, Beutelsbach, Germany) to the anterior segments. Fluorescent images were acquired. In whole eyes, the same protocol was used but without an anterior chamber exchange.

Parameters analyzed

Fluorescent images were captured using a stereomicroscope (Olympus SZX, Olympus K.K., Tokyo, Japan) equipped with a CoolLED pE-300 (CoolLED Limited, London, UK) white illumination unit and an 0.5x objective lens. Canalograms were processed with the Olympus cellSens Dimension 2.3 software (Olympus K.K., Tokyo, Japan). Images were recorded in a time-lapse every 30 seconds for 20 minutes with a resolution of 680 x 510 pixels in 8-bit grayscale. Exposure time was set to 57.14 ms for fluorescein and 344.80 ms for Texas red.

The canalograms were analyzed for filling times of each quadrant: nasal-superior (NS), nasal-inferior (NI), temporal-superior (TS) and temporal-inferior (TI). Filling time was defined as the time elapsed between the first visualization of the dye in the episcleral veins and their complete filling.

Histology

Sagittal specimen sections were obtained and fixed with 4% paraformaldehyde in PBS for 24 hours. After rinsing them three times in PBS, they were embedded in paraffin, sectioned at 6-micron thickness, and stained with hematoxylin and eosin.

Statistical analysis

Data was analyzed using SPSS Statistics (Version 26, IBM, New York, USA). Filling times were reported in seconds. We calculated means and standard deviations. A Shapiro-Wilk test was deployed to check for a normal distribution of acquired filling times. We used a paired, one-tailed t-test and Wilcoxon signed-rank test to compare the mean values of the filling times before and after freeze-thaw. The one-way analysis of variance (ANOVA) was run to compare filling times between quadrants. For all our statistical analyses, a p-value of 0.05 or less was considered statistically significant. A post-hoc power analysis was carried out using G*Power (Version 3.1.9.7., Heinrich Heine University, Düsseldorf, Germany).

Results

A total of 28 eyes were included in the analysis consisting of 16 right eyes and 12 left eyes. There were six eyes in each of A_{CO} , W_{CO} , and W_{FT} and ten eyes in A_{FT} . Within the control groups, A_{CO} and W_{CO} , there were no significant differences between the quadrant filling times (all $p > 0.05$)²⁰. Additionally, both dyes showed similar filling times within each quadrant (Table 1, all $p > 0.05$).

Figure 1 shows the filling times of anterior segments pre- and post-freeze thaw (A_{FT}). In these eyes, quadrant TS tended to have a faster average filling time than the other three quadrants, both pre (270 ± 136 s) and post freeze-thaw (441 ± 371 s), but without reaching statistical significance ($p > 0.05$). Moreover, freeze-thaw did not cause a significant change in the filling times of any quadrant ($p > 0.05$).

Figure 2 depicts the filling times before and after freeze-thaw in W_{FT} eyes. There was no significant difference in the filling times between quadrants ($p = 0.401$). TS showed the shortest

Table 1. Filling times of control groups with fluorescein and Texas red.

Group	Location	Fluorescein	Texas Red	p-value
A_{CO}	TS	465.0 $\pm$ 406.8	510.0 $\pm$ 371.3	0.426
	TI	375.0 $\pm$ 130.8	606.0 $\pm$ 542.7	0.362
	NS	306.0 $\pm$ 250.5	726.0 $\pm$ 425.5	0.113
	NI	300.0 $\pm$ 219.1	330.0 $\pm$ 280.4	0.430
W_{CO}	TS	515.0 $\pm$ 356.7	250.0 $\pm$ 52.5	0.059
	TI	475.0 $\pm$ 382.0	685.0 $\pm$ 417.2	0.195
	NS	456.0 $\pm$ 291.2	636.0 $\pm$ 489.5	0.446
	NI	465.0 $\pm$ 278.0	490.0 $\pm$ 354.8	0.458

TS = temporal superior, TI = temporal inferior, NS = nasal superior, NI = nasal inferior, A_{CO} = perfused anterior segment dye controls, W_{CO} = perfused whole eye dye controls.

Figure 1. Comparison of filling times before and after freeze-thaw in anterior segments. TS = temporal-superior, TI = temporal-inferior, NS = nasal-superior, NI = nasal-inferior.

Figure 2. Comparison of filling times before and after freeze-thaw in whole eyes. Comparison of filling times before and after freeze-thaw in whole eyes TS = temporal-superior, TI = temporal-inferior, NS = nasal-superior, NI = nasal-inferior, * = significant.

filling time before freeze thaw with an average of 162 ± 101 s. The filling time of this quadrant significantly increased after freeze-thaw (355 ± 175 s, $p = 0.002$). None of the other W_{FT} quadrants showed a significant change in filling times after freeze-thawing.

Figure 3 depicts the canalogram of an A_{FT} eye. In this eye, the post-freeze-thaw canalogram demonstrated additional temporal outflow channels that were not visible before (TI) or only partially filling (TS).

Figure 4 shows canalograms of W_{FT} eye pre- and post- freeze-thaw. Visually, both images show a similar filling pattern after 20 minutes of perfusion. In this case, fluorescence was captured mainly in the nasal episcleral veins.

Histological analyses demonstrated unchanged trabecular beams but without intact trabecular meshwork cells (Figure 5). Compared to control eyes, there were no noticeable changes to the tissue surrounding the angular aqueous plexus. The lumina of this plexus, including larger, Schlemm's canal like-segments remained intact.

The post-hoc power analysis of the filling times pre- and post- freeze-thaw for each group is presented in Table 2. In general, results revealed a low power for most of the quadrants. Within the experimental groups, the TS quadrant comparison had the highest power with values of 0.5 and 0.75 in A_{FT} and W_{FT} respectively. All other quadrants in both groups had a power below 0.3.

Discussion

We have developed a freeze-thaw protocol to decellularize anterior segments and to obtain a three-dimensional matrix to transplant modified trabecular meshwork (TM) cells or other cells

under study^{16,21}. This approach might generate *ex vivo* models for genetically altered, glaucomatous TM cells or primary cells harvested in excisional *ab interno* trabeculectomy^{21–23}. Surprisingly, freeze-thawing did not alter the vascular drainage spaces qualitatively in a significant way. Rather, dyes remained mostly intravascularly within the time observed without any noticeable differences on average as detectable with these methods. Fluorescein has a molecular size of 376 Da and sulforhodamine 101 acid chloride a size of 625 Da, small enough to pass through the TM relatively quickly, yet large enough to be confined to the intravascular space for minutes^{24–26}. Histologic analyses also revealed a conserved TM structure^{16,21}. It is not unreasonable to assume here that the extracellular matrix also remains unchanged as we have previously studied the effects of freeze-thawing on this structure¹⁶. This preserved scaffold along with the unchanged outflow vessels suggests freeze-thawing as a technique to produce realistic seeding matrices for trabecular meshwork transplantation.

Because freeze-thaw had previously been shown to cause an IOP drop of about 30% in porcine eyes¹⁶, we expected to see faster outflow throughout the 360 degrees of angular aqueous plexus of both anterior segments and whole eyes. Surprisingly, this was also not the case. The only quadrant in which there was a statistically significant difference was the temporal superior quadrant of whole eyes, and we observed an increase rather than a decrease in filling time. It is possible that freeze-thawing causes relatively small changes per trabecular meshwork area and that our study missed those due to the large standard deviation. However, these small changes might have a large cumulative impact and lower IOP¹⁶ because they occur over the entire circumference. For instance, flow in TI and NS had a lower average flow time after freeze-thaw compared to before, but without reaching statistical significance. Moreover, due to their moderate molecular size, movement of fluorescein

Figure 3. Freeze-thaw treated anterior segment cultures (A_{FT}) canalogram before (A) and after (B) freeze-thawing. Example of perfused anterior segment culture, in which outflow channels that were not functional become visible after freeze thaw (white arrows). TS = temporal-superior, TI = temporal-inferior, NS = nasal-superior, NI = nasal-inferior.

Figure 4. Freeze-thaw treated whole eyes (W_{FT}) canalogram before (A) and after (B) freeze-thawing. After a 20-minute infusion with fluorescein, a fluorescent signal was detected in the nasal-superior (NS) quadrant (A). This outflow pattern experienced only minor changes (white arrows). The shadow of the infusion needle (N) is visible on the right (temporal) side. TS = temporal-superior, TI = temporal-inferior, NS = nasal-superior, NI = nasal-inferior.

Figure 5. Sagittal histology sections comparing the angular aqueous plexus in control (A) and freeze-thaw ablated eyes (B). The TM beams and SCLS remain intact after freeze-thaw. TM = trabecular meshwork, SCLS = Schlemm's canal-like segments (red arrows).

Table 2. Post-hoc power analysis for every quadrant.

Quadrant	Power A _{FT}	Power W _{FT}	Power A _{CO}	Power W _{CO}
TS	0.5	0.75	0.08	0.08
TI	0.25	0.11	0.18	0.3
NS	0.11	0.14	0.67	0.13
NI	0.15	0.14	0.07	0.07

TS = temporal-superior, TI = temporal-inferior, NS = nasal-superior, NI = nasal-inferior, A_{CO} = perfused anterior segment dye controls, W_{CO} = perfused whole eye dye controls, A_{FT} = freeze-thaw treated anterior segment cultures, W_{FT} = freeze-thaw treated whole eyes

and sulforhodamine through an intact trabecular meshwork might not be fundamentally different from movement through remnants of disrupted trabecular meshwork cells or their debris²⁷. Additionally, our methods might miss diffuse fluid movements directly through the sclera that is akin to uveoscleral outflow.

We observed a longer post freeze-thaw filling time in the temporal superior quadrant of whole eyes. It is likely that intraocular tissues that are present in whole eyes, but are missing in anterior segments²⁸, were disrupted by the freeze-thawing cycles. Their debris might have caused the delayed filling²⁷, in particular iris pigment^{6,29,30}. Pigment can lead to a slow increase of IOP over hours to days⁶ while larger debris from cell death can have a more immediate effect on the trabecular meshwork²⁷. However, previous research has shown successful repopulation after using a saponin detergent for decellularization³¹. Therefore, we assume a similar outcome after a less invasive cell depletion by our freeze-thaw method.

One limitation is that we did not measure IOP because a goal of this study was to assess freeze-thawing in anterior segment cultures in comparison to whole eyes; however,

the latter cannot easily be connected to a pressure transducer. Another limitation is that the eyes were not incubated for three days as we have done previously¹³. Due to the compression mount, outflow in anterior segments perfusion cultures is initially impaired, but this effect vanishes within less than three days of culture¹⁶. Finally, the 28 eyes used in this study were only able to provide a moderately low testing power to detect an outflow difference, providing further evidence that any flow difference caused must not be large.

In conclusion, we found no major outflow differences caused by freeze-thaw treatment of anterior segments or whole eyes. These results validated freeze-thawing as a method to generate a three-dimensional seeding matrix without losing distal outflow tract vessels.

Data availability

Underlying data

Figshare: RawData_FreezeThaw.csv, <https://doi.org/10.6084/m9.figshare.14610579.v1>²⁰

The project contains the following underlying data:

- RawData_FreezeThaw.csv (raw data of freeze-thaw in porcine eyes, filling times assessed using fluorescent analogography before and after freeze thaw)

Figshare: Original Images FT, <https://doi.org/10.6084/m9.figshare.14769888.v1>³².

- Original, unedited image files underlying our results and figures

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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VijayKrishna Raghunathan

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This is an interesting study by Verma-Fuehring et al documenting the impact that freeze-thawing has on outflow time across the TM in porcine eyes. The study is largely conducted in a simple and effective manner, but could perhaps be improved with some clarifications.

1. The authors utilized a slow method of freezing anterior segment and whole globes through freezing in -80C. Due to variations in tissue thickness and permeability differences, perhaps a quicker freezing method could have been utilized to minimize such artifacts. e.g. rapid freezing using Liquid N2. Can the authors please clarify the choice of method used?
2. After freeze-thaw, the authors choose to demonstrate through H&E staining and canalogram that fluid filling rates were unchanged. Subsequently they infer that this implies no structural changes were observed in the TM and hence the model may be adequate. However, no evidence to demonstrate changes in ECM composition (if any, or lack thereof) and organization is provided. This is particularly important since free-thaw techniques generally influence GAGs and other secretory proteins in tissues, at least as reported in non-ocular literature. This becomes even more important considering ECM composition and GAGs help maintain cellular phenotype and can influence differentiation. Can the authors please comment.
3. Freeze-thaw indeed can cause cell lysis and loss of cells, as evident in their H&E staining. However, can the authors comment how one might go about removing debris and washing off porcine immunogenic/antigens that when human cells may be transplanted (as indicated for intended application) may not react to?
4. Can the authors comment if they have attempted a more localized free-thaw technique (e.g. a cryo probe) that may not affect the integrity of the entire tissue and if this may be more favorable for such a model?
5. Should the authors have the images, the manuscript may benefit in inclusion of a kymograph-like image demonstrating the appearance of the fluorescein dye to show fill

timings.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Trabecular meshwork mechanobiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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Christian van Oterendorp

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The authors present a study on the influence of freeze-thaw decellularisation on the aqueous outflow tract in porcine eyes. They hypothesised that freeze-thaw would result in a higher outflow rate and changes to outflow pattern.

The study was designed as a longitudinal study, which was achieved by using two different fluorophores to conduct the experiments pre- and post-freeze-thaw in the same eyes. Filling time was used as surrogate parameter for the volumetric flow rate. This was supplemented

by qualitative histological studies of the angular aqueous plexus.

The experimental setup is sound and the data is presented in a well structured fashion. To increase the transparency of the data the authors may contemplate choosing scatter plots with connecting lines for each pair of pre-post-data instead of bar and whisker plots. Thus, the reader would be able to see the change of filling time for each individual eye.

While the main focus of data analysis was on the filling time, the authors show only 2 examples of changes of the outflow pattern (fig. 3+4). Quantifying these changes systematically would be desirable, because figure 3 suggests that the total cross sectional area of the aqueous drainage system has increased. Thus, the total volumetric flow rate of the eye (at constant perfusion pressure) could have changed despite a constant filling time of each individual vessel. The observation presented in figure 3 should also be further discussed regarding possible mechanisms of opening previously closed outflow channels.

One last point in regards the experimental setup: The authors used a 30G needle to perfuse the anterior chamber. Depending on the outflow rate of the eyes, the small internal diameter of the needle may render the needle an element of significant flow resistance, which in turn may result in an IOP lower than the hydrostatic pressure applied with the 20ml syringe at 20 cm height.

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Yes

Is the study design appropriate and is the work technically sound?

Yes

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Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: glaucoma, aqueous drainage

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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