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Expression of Na^+/K^+ATPase and changes of corneal endothelium cell density after phacoemulsification with hypothermic perfusion

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ABSTRACT

Background: Intraocular hypothermic perfusion can be used as an alternative to reduce corneal endothelial cell damage in phacoemulsification procedures. This study aimed to analyze the effect of hypothermic intraocular perfusion on cell density and expression of the Na^+/K^+-ATPase on corneal endothelial cells after exposure to ultrasonic phacoemulsification energy.
Methods: Sixteen New Zealand white rabbits (n=16 eyes) were randomly divided into two groups and exposed to phacoemulsification ultrasonic energy. The control group received room-temperature Intraocular Balanced Salt Solution (BSS) hyperthermic perfusion (24ºC) and the treatment group received low-temperature intraocular BSS hypothermic perfusion (4ºC). Corneal endothelial cell density (ECD) was measured before and 1 day after surgery using a specular microscope. On the first postoperative day, the expression of Na^+/K^+ATPase was examined using immunohistochemical antibody staining. Data were analyzed using SPSS version 26 for Windows.
Results: There was no statistically significant difference in the expression of corneal endothelial Na^+/K^+-ATPase between the control and treatment groups (p=0.053). There was no significant difference in corneal endothelial cell density changes between the control and treatment groups (p=0.115). There was no correlation between changes in cell density and corneal endothelial Na^+/K^+ATPase expression in the control and treatment groups (p=0.216).
Conclusion: The effect of intraocular hypothermic perfusion on phacoemulsification procedures remains controversial. Endothelial cell density decreased in hypothermic perfusion after phacoemulsification and Na^+/K^+ATPase expression was not significantly affected by hypothermic perfusion.

Keywords: Phacoemulsification, Endothelial Cells Density, Na^+/K^+ATPase.
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INTRODUCTION

Phacoemulsification is a revolution in cataract surgery and is widely recognized as the most effective method compared to other cataract surgery techniques. The phacoemulsification technique employs ultrasonic energy to generate both thermal and non-thermal effects.[1] Thermal effect causes the generation of heat energy, which causes incision wound burning, corneal edema, anterior chamber (AC) inflammation, and corneal decompensation.[2] Corneal endothelial cells may exhibit morphological changes and a decrease in cell density following phacoemulsification, resulting in a loss of corneal transparency and reduced visual acuity.[3]

Around 285 million people worldwide are estimated to have a visual impairment, with cataracts accounting for 33% of the cause.[4] Even sophisticated phacoemulsification procedures can result in 4.2% - 16.7% endothelial cell loss, which is related to the degree of trauma during surgery and can result in decreased postoperative visual acuity.[5] Thermal damage after phacoemulsification can result in endothelial cell detachment, changes in endothelial cell density (ECD), and immunohistochemically indicate cell disorganization.[6]

Corneal hydration occurs as a result of a balance between the movement of aqueous humor from the corneal endothelium to the stroma and then being pumped out of the stroma.[7] The pump's activity in removing fluid from the cornea is determined by the number of endothelial cells and the effectiveness of each cell's pump function.[7] The expression of Na^+/K^+ATPase on the basolateral membrane of corneal endothelial cells is primarily responsible for the endothelial cell's pump function.[8] Phacoemulsification surgery is the process that uses intraocular irrigation fluid. Balance Salt Solution (BSS) is a common irrigation fluid whose composition is similar to the physiological conditions of aqueous humor.[2] Hypothermia is a protective factor that can increase the body's tolerance to ischemia and hypoxia, indicating that the corneal structure can be more stable in hypothermic perfusion conditions.[9]

This study was an in vivo study on white rabbit eyes exposed to ultrasonic phacoemulsification energy and treated with intraocular hypothermia irrigation

at 4°C and 24°C at room temperature. The expression of the Na^+/K^+ATPase was analyzed through anatomical pathology examination with immunohistochemical antibodies.

METHODS

This study was a true experimental study on New Zealand white rabbits (*Oryctolagus cuniculus*) with pre-test and post-test control group design to evaluate the effect of hypothermic intraocular perfusion on corneal endothelial cell density and post-test only control group design to evaluate the expression of Na^+/K^+ATPase after phacoemulsification. *Oryctolagus cuniculus* was divided into two groups: the control group received U/S power phacoemulsification with intraocular irrigation fluid at 24°C room temperature, and the treatment group received U/S power phacoemulsification with hypothermic intraocular irrigation fluid at 4°C.

The inclusion criteria for this study were *Oryctolagus cuniculus* rabbits aged 12-15 weeks with a body weight of 2.5-3.0 kg.[9] The rabbit appeared to be active and healthy, and the eyes were in good condition on external examination by a veterinarian. Exclusion criteria included rabbits declared by a veterinarian to have a disease or the potential to transmit the disease during the study and rabbits with corneal endothelial cell morphological abnormalities. The dropout criteria were sick, dead rabbits, or complications such as corneal perforation, vitreous prolapse, infection, and bleeding during and after surgery.[10]

Sixteen *Oryctolagus cuniculus* rabbits (16 eyes) were included in the inclusion criteria and divided into two groups, each consisting of eight rabbits (8 eyes). The right eye received 0.5% tetracaine hydrochloride topical anesthetic eye drops and pupillary dilation with 2.5% phenylephrine hydrochloride and 1% cyclopentolate hydrochloride. Anesthetic injections of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg) were administered for general anesthesia.[10] A clinical examination was performed preoperatively and one day after the procedure. A handheld slit lamp was used to examine the anterior segment and endothelial cell density was examined using a specular microscope (NIDEK CEM-530) on the central part of the cornea.[11]

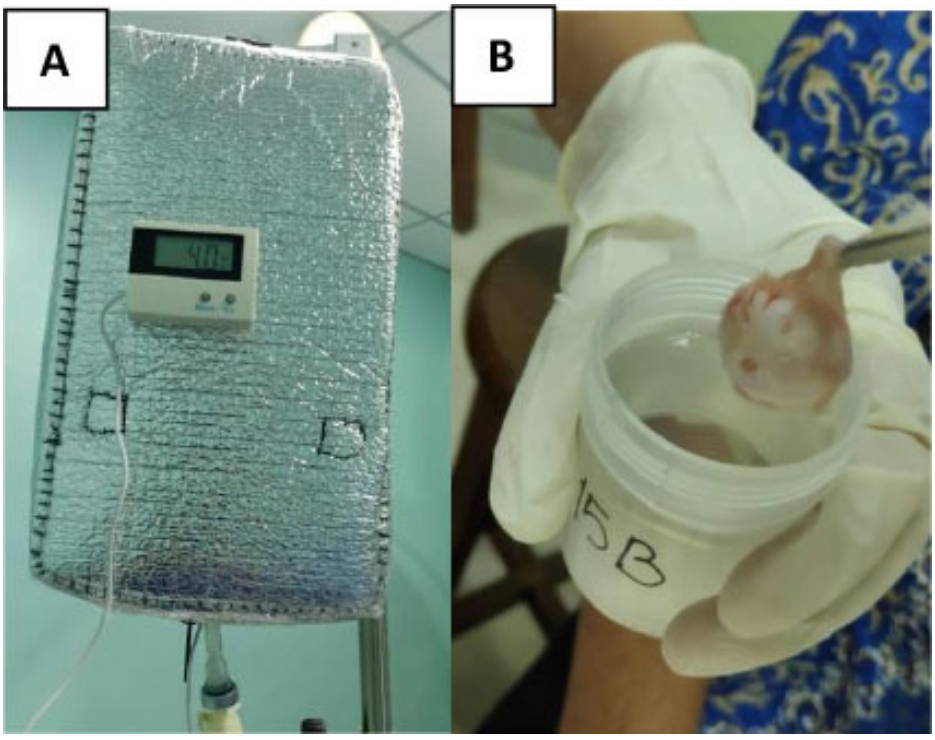

Figure 1. (A) Coolbox for hypothermic BSS and (B) Rabbit eyeball after enucleation.

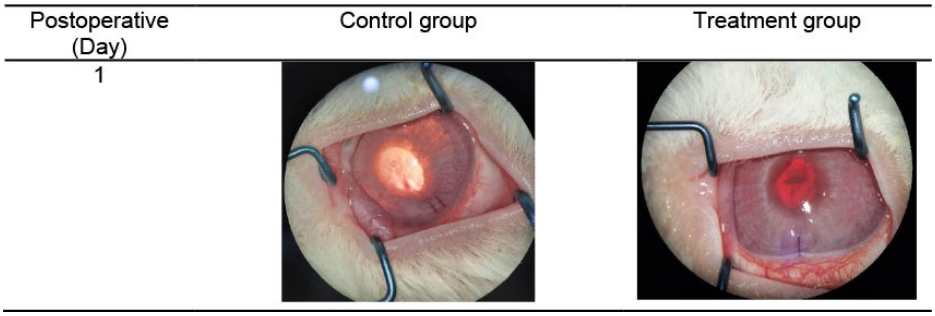

Figure 2. Anterior segment day 1 post phacoemulsification.

Intraocular BSS temperature was regulated in two conditions: at room temperature of 24°C for the control group and 4°C at hypothermic temperature for the treatment group (Figure 1). The hypothermic BSS was kept in a refrigerator at 4°C for at least 12 hours before being used.[12] During the surgery, the BSS at 4°C was kept in a coolbox containing ice gel to maintain a stable temperature. The digital thermometer probe was placed in the coolbox to measure the BSS temperature during surgery.[13]

Disinfection of the eyelids using 10% povidone-iodine and then the operating field is narrowed with a sterile eye drape. Disinfection of the eyeball using 5% povidone-iodine and then rinsing. Phacoemulsification was performed on a NIDEK CV 9000R machine, and the surgery was performed using a technique modified by Nemet *et al.*[14] The corneal incision was made in the superotemporal area with a 2.75 mm keratom. The bevel phaco tip position faces upwards in the anterior chamber towards the endothelium. The phacoemulsification parameter was set at 70% burst mode panel strength, bottle height 90 cm, aspiration flow rate 25 ml/minute, and a total duration of 5 minutes (10 seconds power on and 10 seconds power off).[14] The corneal incision was closed using sutures with 10.0 nylon thread.[9] The surgery was

performed by a single eye surgeon with a visualization microscope (Appasamy Operating Microscope Brilliant Advent). Postoperatively, rabbits were given Levofloxacin 0.5% non-preservative antibiotic eye drops 4 times daily.[10] On the first postoperative day, the density of corneal endothelial cells was measured. All rabbits were then terminated and enucleated. The cornea was separated from the eyeball and fixed using 10% buffered formaldehyde for histopathological examination.[11]

Corneal endothelial cell density was measured using a specular microscope (NIDEK CEM-530). Data collection was done in the center of the cornea three times each, and the average value was calculated. Corneal endothelium was measured before treatment and then one day after treatment using the same tools and techniques. This procedure is performed with rabbits under general anesthesia. Changes in cell density were assessed from the difference in the number of cells during the examination before and after surgery.[10] The corneal samples performed immunohistochemical examination with Na^+/K^+ATPase antibodies (GTX30202, GeneTex Lab, China). Na^+/K^+ATPase cells were stained as light brownish to chocolate brown under a light microscope. A semi-quantitative method using immunoreactivity score (IRS) assessment in 3 fields of view. The pathologist conducted this at x400 magnification and there was only one pathologist to analyze the Na^+/K^+ATPase cells. To reduce bias, this technique was carried out by pathologists on serially numbered slides in a blinded manner using an Olympus microscope (Cx51) equipped with an Olympus camera using SIS software (Japan, Tokyo).[10]

The collected data were analyzed using SPSS version 26 for Windows. Comparison of Na^+/K^+ATPase expression and changes in endothelial cell density between the two groups were analyzed using an independent t-test (normally distributed data) or the Mann-Whitney test (data not normally distributed). The correlation between Na^+/K^+ATPase expression and changes in endothelial cell density was analyzed using the Pearson test (normally distributed data) or Spearman's test (data

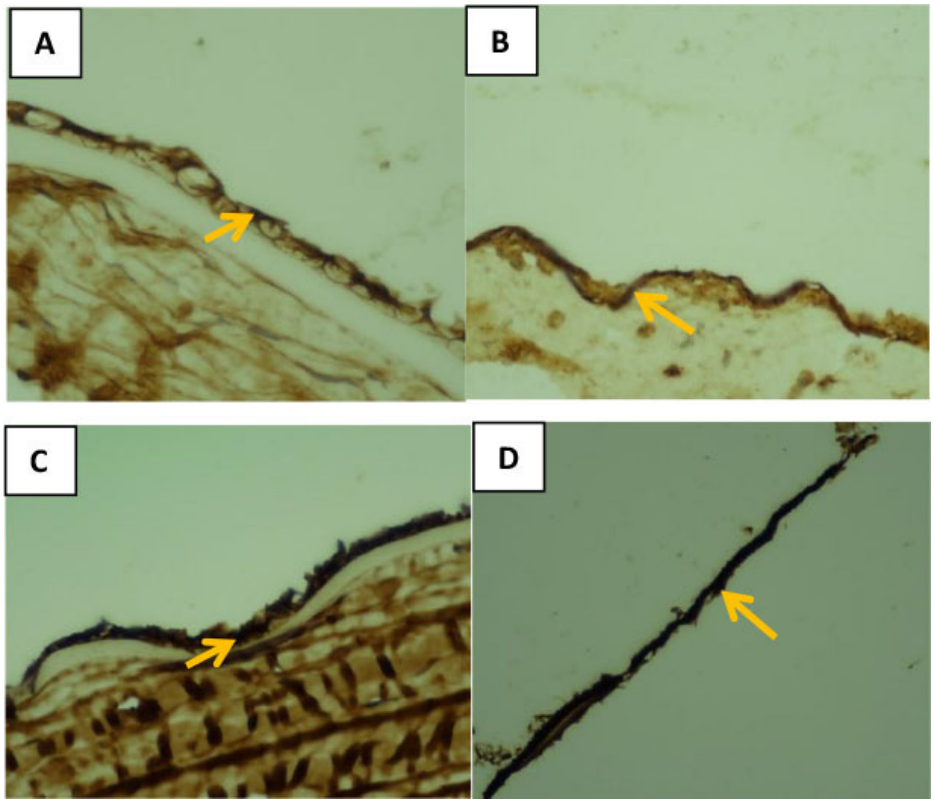

Figure 3. Expression of Na^+/K^+ATPase by examination of Na^+/K^+ATPase antibodies viewed with a 400x magnification light microscope. (A) and (B) control group, (C) and (D) treatment group.

Table 1. Corneal Endothelial Cell Density (ECD) Distribution.

Group	Endothelial Cell Density		P	Density Change
	Pre-Phacoemulsification mean ± SD	Post Phacoemulsification mean ± SD		
Control	2759.08 ± 188.65	2581.75 ± 343.93	0.033[b]	204.67 ± 154.43
Treatment	2518.50 + 212.24	2073.00 ± 429.62	0.023[b]	486.92 ± 380.30
p	0.031[a]	0.020[a]		0.115[c]

a=independent t-test; b=paired t-test; c=Mann-Whitney test

Table 2. Expression of Na^+/K^+-ATPase.

Group	N	Expression of Na^+/K^+-ATPase				p
		Mean	SD	Minimum	Maksimum	
Control	8	6.75	1.28	5	9	0.053
Treatment	8	8.00	1.07	6	9	

SD: Standard Deviation; *Independent T-Test; statistically significant if p-value less than 0.05.

Table 3. Correlation of Na^+/K^+-ATPase expression with changes in cell density after phacoemulsification.

Variable	p
	ECD Change
Na^+/K^+-ATPase	0.216

*Spearman Correlation Test: statistically significant if p-value less than 0.05

not normally distributed). The p-value < 0.05 indicates statistically significant data.

RESULTS

This study used an experimental unit of 16 corneas of New Zealand white rabbits weighing 2.5-3.0 kg and aged 12-15 weeks obtained from rabbit farms under the supervision of a veterinarian. The study was conducted at the Faculty of Veterinary Medicine, Airlangga University, Surabaya. All rabbits were alive, healthy, and had no complications during and after the phacoemulsification procedure.

Examination of the anterior segment before phacoemulsification was found to be within normal limits. Endothelial cell density before and after phacoemulsification was found to be normally distributed between the control and treatment groups and a significant difference was found between the two (table 1).

On day 1 after phacoemulsification, the anterior segment was examined using a handheld slit lamp and edema was more severe in the control group than in the treatment group and no infection was found in either group. The mean value of ECD in the control group (2759.08 ± 188.65) and in the treatment group (2518.50 ± 212.24) before phacoemulsification with p-value = 0.031 (p < 0.05, a significant difference was found). The mean value of ECD in the control group (2581.75 ± 343.93) and the treatment group (2073.00 ± 429.62) after phacoemulsification also found a significant difference with p-value = 0.020 (p < 0.05). Cell density before and after the intervention in the control group was analyzed using a paired t-test, p = 0.033 (p 0.05), indicating a significant difference between cell density before and after the procedure in the control group. Meanwhile, the cell density before and after the intervention in the treatment group was analyzed using a paired t-test, and the result was p = 0.023 (p 0.05), indicating a significant difference between the cell density before and after the intervention in the treatment group. Changes in ECD in the control group (204.67 ± 154.43) and the treatment group (486.92 ± 380.30) showed that the results were not statistically significant between the two with p = 0.115 (p > 0.05) (Table 1).

The expression of Na^+/K^+ATPase was examined using immunohistochemical staining (IHC) with Na^+/K^+ATPase antibody according to the percentage of endothelial cells expressing Na^+/K^+ATPase under a light microscope with 400 times magnification. Interpretation of the results was assessed based on the immunoreactive score (IRS) in 3 fields of view. A brownish color identifies endothelial cells that express Na+/K+ATPase on the cell membrane. The treatment group was paler in color than the control group in the IHC examination. Yellow arrows indicate a single layer of endothelial cells stained with Na+/K+ATPase antibody (Figure 3).

According to table 2, the control group's mean value of Na^+/K^+ATPase expression (6.75 ± 1.28) was lower than the treatment group's (8.00 ± 1.07). However, statistical analysis using an Independent T-Test revealed no statistically significant difference between the control and treatment groups (p = 0.053) (Table 2).

The distribution of Na^+/K^+ATPase expression and changes in endothelial cell density after phacoemulsification were not normally distributed in both groups, with the value of Na^+/K^+ATPase expression being p = 0.083 (p > 0.05) and a change in cell density was p = 0.002 (p < 0.05).

Spearman's test was used to analyze the correlation between Na+/K+ATPase expression and changes in endothelial cell density, with p = 0.216 (p > 0.05). This indicates no correlation between Na+/K+ATPase expression and corneal endothelial cell density changes after phacoemulsification (Table 3).

DISCUSSION

Our study was to analyze the effect of intraocular hypothermic perfusion on cell density and Na^+/K^+ATPase expression of corneal endothelial cells after exposure to ultrasonic phacoemulsification energy. Cataract extraction using the phacoemulsification technique can alter the density of corneal endothelial cells by significantly reducing the number of cells postoperatively. A study conducted by Pardasani et al., showed that endothelial cell density decreased significantly on day 1 postoperatively and continued to decline until 3 months postoperatively. The damaged endothelial cells do not have the ability to regenerate.[15] This is consistent with the findings of our study, in which endothelial cell density decreased on day 1 postoperatively in both the control and treatment groups, with p = 0.033 in the control group and p = 0.023 in the treatment group. The results showed that ultrasonic energy phacoemulsification significantly reduced endothelial cell density.

The corneal endothelium can be effectively protected from thermal damage caused by phacoemulsification by using hypothermic intraocular irrigation fluid.[9] Other studies have suggested that hypothermia slows down tissue metabolism rate and enzymatic reactions.[16] This statement is consistent with a study by Wan et al., in which corneal endothelial cells in the 4°C group had a higher endothelial cell density than the 24°C room temperature group on day 1 postoperative. However, there was no significant difference in endothelial morphology between the two groups on the 7th postoperative day.[9] According to the study, the temperature of the anterior chamber in the eyes of experimental rabbits was the same when irrigation fluid was administered at 4°C and 10°C. This can occur due to several factors, including the difference between the temperature of 4°C and 10°C is not clear enough, especially when hypothermic fluid flows into the anterior chamber of the eye through a long tube and its temperature can change during this process.[9] Data from another study conducted by Uthaisang et al.,

showed that BSS at 8°C was not harmful to cell survival and may be more effective in protecting the endothelium than BSS at 25°C.[2]

Some of these studies' results contradict our study's results, which found that the density of endothelial cells treated with a hypothermic temperature of 4°C experienced a significant decrease in the number of cells. However, there was no statistically significant difference in the changes in endothelial cell density between the control group and the post-phacoemulsification treatment with hypothermic intraocular perfusion. This indicates that intraocular hypothermic perfusion compared to intraocular room temperature in the phacoemulsification procedure, has no effect on changes in endothelial cell density on the 1st postoperative day. Our study is similar to that of Joussen et al., who showed that in vivo experimental studies using intraocular irrigation fluid normothermic 23°C and hypothermic 5°C in rabbit eyes did not induce significant changes in corneal endothelial morphology.[17] This study agrees with the research data of Yagoubi *et al* who reported that intraocular irrigation fluid at a lower temperature than room temperature had no effect on corneal parameters and did not prevent postoperative corneal burns.[17] Yagoubi *et al* stated that perfusion of the anterior chamber of the rabbit's eye caused an increase in corneal pachymetry caused by exposure to the composition and intraocular irrigating fluid but not by the temperature of the irrigating fluid.[18]

A study by Praven et al., showed that there was no statistically significant change in endothelial cell density in the control group at 23°C and 10°C treatment and there was no significant difference in anterior segment inflammation on day 1 postoperatively.[12] The study results showed that the low temperature of the intraocular irrigation fluid had no effect when compared to room temperature on corneal parameters and inflammation, so the conclusion was that the use of refrigerated BSS had no effect and benefit on the outcome of phacoemulsification.[12] The optimal temperature of intraocular irrigation fluid during surgery, especially the phacoemulsification procedure, remains controversial.[17] The advantage of using hypothermic irrigation fluids during the phacoemulsification process remains questionable. Several studies suggested that the ionic composition in irrigating fluid tends to be more influential on the outcome of phacoemulsification and that there was no effect of temperature in intraocular irrigation fluid.[17]

The corneal endothelium is a non-mitotic tissue that plays an important role in maintaining the stability of corneal transparency. The corneal endothelium maintains a balance between the flow of aqueous humor into the stroma and the pumping of aqueous humor from the stroma into the eye's anterior chamber. The decrease in the number of endothelial cells associated with age in healthy corneas is compensated by increased activity of Na^+/K^+ATPase, a basic function of the endothelial pump.[19] Oxidative stress and inflammatory response caused by phacoemulsification procedures can reduce nitric oxide release and cause endothelial dysfunction. Research conducted by Kösekahya et al., reported that ocular inflammation has a negative effect causing endothelial dysfunction.[19]

Our study was to evaluate the presence of Na^+/K^+ATPase expression in corneal endothelial cells after intraocular hypothermic perfusion in the phacoemulsification procedure using IHC antibody staining. The study by He et al., used antibody staining similar to ours. In this study, there were differences in color intensity between endothelium, Descemet's membrane, and stroma, where the endothelial cells stained with a thicker brown color. This indicates that antibody staining reacts specifically as an interpretation of Na^+/K^+ATPase expression in the corneal endothelium.[20] The results of staining of Na^+/K^+ATPase antibodies in the control group were paler than in the treatment group, which could indicate that Na^+/K^+ATPase was still more expressed by endothelial cells in the treatment group that received hypothermic intraocular perfusion during phacoemulsification but the difference between the two groups not statistically significant.

The study by Hatou et al., showed that the density of Na^+/K^+ATPase on the corneal endothelium was increased in eyes with moderate corneal guttae, indicating a low endothelial cell density. The density of Na^+/K^+ATPase increased initially and then gradually decreased. These findings suggest that certain conditions can cause a compensatory increase in the density and function of the Na^+/K^+ATPase pump.[7,20,21] This is consistent with our findings, which showed that corneal endothelial cell densities were reduced in both the control and post-phacoemulsification groups. However, the treatment group had a higher mean value of Na+/K+ATPase expression than the control group, which could be a compensatory mechanism for the Na+/K+ATPase, though the difference between the two groups was not statistically significant.

In our study, there was no correlation between Na^+/K^+ATPase expression and changes in endothelial cell density on intraocular hypothermic perfusion after phacoemulsification surgery. This could be due to the expression of Na^+/K^+ATPase, which was examined histologically while endothelial cell density was determined clinically. Furthermore, the compensatory factor of the endothelial Na^+/K^+ATPase can influence its work activity, preventing it from being affected by changes in corneal endothelial cell density.

There are some limitations in our study. Clinical analysis was only observed in the initial phase, namely day 1 post-treatment. This study used a specular microscope to examine the corneal endothelium only in the central area of the cornea, so the cell density condition on the entire cornea surface cannot be explained. The thermometer probe is only placed in the coolbox when the BSS is hanging and is not set in the anterior chamber, so it cannot precisely detect changes in temperature in the anterior chamber during phacoemulsification. Further research is needed with a more extended research period long and serially to evaluate significant differences in corneal endothelial cells on the effect of hypothermic perfusion in cell density and Na^+/K^+ATPase expression after phacoemulsification. Need to use a method of examining the corneal endothelium that is not only centrally but on the entire cornea surface so that the condition of cell density and Na^+/K^+ATPase expression can

be more precisely identified throughout the corneal surface. A thermometer probe is placed not only in the coolbox when the BSS hangs but also in the anterior chamber so that temperature changes in the anterior chamber can be known with certainty during phacoemulsification.

ETHICAL CLEARANCE

Ethics approval has been approved by the Ethics Committee for Basic and Clinical Science Research, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia (No. 2.KEH.018.03.2022).

CONFLICT OF INTEREST

There is no conflict of interest regarding the manuscript.

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AUTHOR CONTRIBUTIONS

All authors equally contribute to the study from the conceptual framework, data acquisition, and data analysis until reporting the study result through publication.

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