Expression of Superoxide Dismutase-1 (SOD-1) and changes of corneal endothelial morphology after phacoemulsification with hypothermic perfusion

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ABSTRACT

Background: Hypothermic perfusion can be used as adjunctive therapy to reduce corneal endothelial cell damage during phacoemulsification procedures. This study aims to evaluate the effect of hypothermic perfusion on the expression of Superoxide Dismutase-1 (SOD1) and morphological changes in the corneal endothelium after ultrasound energy of phacoemulsification exposure.

Methods: Sixteen New Zealand white rabbits (n=16 eyes) were randomly divided into two groups and exposed to ultrasound energy of phacoemulsification. The control group was administered room temperature (RT) (24°C) Balanced Salt Solution (BSS) intraocular perfusion, and the treatment group was administered hypothermic (4°C) BSS intraocular perfusion. Coefficient of Variation (CV) and hexagonality were measured before and one day after surgery with specular microscopy. The expression of SOD1 was examined by immunohistochemistry staining. Data were analyzed using SPSS version 26 for Windows.

Results: The result showed that the SOD1 expression was significantly lower in the hypothermic perfusion group compared with the control group (p=0.024, p<0.05). There were no significant differences between the two groups in CV changes (p=0.494, p>0.05) and hexagonality changes (p=0.916, p>0.05). There was no correlation between the expression of SOD1 and change in CV (p=0.188, p>0.05) or the expression of SOD1 and change in hexagonality (p=0.763, p>0.05).

Conclusion: Hypothermic perfusion suppresses all metabolic processes, including the expression of SOD1 and ROS production. The coefficient of variation and hexagonality as a representative of corneal endothelial morphology changes also decrease with hypothermic perfusion after ultrasound exposure but are not statistically significant. There is no correlation between the expression of SOD1 and changes in CV and hexagonality.

Keywords: Corneal Endothelial, CV, Hypothermic Perfusion, Phacoemulsification, SOD.


INTRODUCTION

Phacoemulsification is an efficient and safe cataract surgery technique.¹ However, phacoemulsification utilizes high-intensity ultrasound energy to cause trauma to the cornea, especially the endothelial layer.¹ Excessive endothelial damage can cause irreversible bullous keratopathy.² Park et al., reported a decrease in corneal endothelial cells of 5.2%-9.1% two months after phacoemulsification and will continue to decline for up to a year.² Bullous keratopathy is a late complication of phacoemulsification and occurs in about 1%-2% of patients after cataract surgery.³

Thermal trauma is caused by excess energy generated by ultrasound energy, while the formation of acoustic cavitation causes non-thermal or mechanical trauma.⁴ Endothelial damage from thermal trauma can be prevented by intraocular perfusion, which forms the anterior chamber, preventing phaco tip friction with the corneal endothelium and cooling the anterior chamber temperature.⁴ Intraocular perfusion initially used Ringer's lactate (RL), then replaced with Balanced Salt Solution (BSS), which has an ionic composition similar to aqueous to aequous to minimize postoperative complications. However, post-phacoemulsification endothelial damage is still found with BSS.⁵

The presence of trauma to the cornea will induce the formation of free radicals, namely hydroxyl radicals and superoxide anions.⁶ Free radicals trigger antioxidant activity to fight free radicals.⁷ Antioxidants that are widely found in corneal tissue and act as the first line against free radicals are SOD.⁷ The imbalance between free radicals and antioxidants causes oxidative stress.⁸ Excessive oxidative stress can cause endothelial cell death, resulting in changes in endothelial cell morphology.⁹ CV and hexagonality are parameters used to represent the endothelial cell morphology during the recovery process after trauma.⁹ CV indicates corneal endothelial dysfunction, while hexagonality indicates endothelial wound healing process.⁹

Hypothermic perfusion can be used as adjunctive therapy to reduce endothelial damage due to phacoemulsification. Wan et al. reported hypothermic perfusion...
could reduce corneal edema and anterior segment inflammation in the early postoperative stage. Jiang et al. reported hypothermic perfusion in uveitis-associated cataracts could inhibit anterior segment inflammation and reduce corneal edema.

The present study aims to assess the effect of hypothermic perfusion on the corneal endothelium during the phacoemulsification process by evaluating SOD1 expression and morphological changes in corneal endothelial cells.

**METHODS**

This study is an experimental study using animals with pre and post-exposure to examine corneal endothelial morphology and the SOD1 expression after exposure to ultrasound energy of the phacoemulsification.

This study was approved by the institutional review board and ethics committee of the Veterinary Faculty, Universitas Airlangga. Healthy male New Zealand white rabbits (*Oryctolagus cuniculus*) without any signs of corneal abnormalities aged 12-15 weeks and weight 2.5-3.0 kg were used in this study. The dropout criteria were unhealthy, dead, and complications such as corneal infection (keratitis), corneal perforation, vitreous prolapse, and bleeding during and after surgery.

Sixteen *Oryctolagus cuniculus* (16 eyes) were included in the criteria and divided into two groups, each consisting of eight rabbits (8 eyes). Subject allocation to the control and treatment groups was performed randomly. The control group was administered room temperature (RT) (24°C) BSS intraocular perfusion, while the treatment group was administered hypothermic perfusion (4°C) BSS intraocular perfusion during exposure to phacoemulsification ultrasound energy.

**Clinical Examination**

An anterior segment examination was performed before and one day after surgery. The analysis of the anterior segment using a handheld slit lamp. Corneal endothelial morphology was analyzed by endothelial CV and hexagonality using a specular microscope (NIDEK CEM-530) in the central cornea. The specular microscope examination was repeated three times for each measurement, and the mean value was used in all calculations.

**Intraocular irrigation fluid temperature setting**

RT BSS was obtained by storing BSS in a room setting at 24°C for at least 12 hours and measured using a room thermometer. BSS was placed in a refrigerator set at 4°C for at least 12 hours to achieve hypothermic BSS (4°C), which was then measured with a thermometer. For BSS to remain at 4°C during the surgery, BSS was put into a cool bag with ice gel inside. A digital thermometer is placed in a cool bag to determine the BSS temperature during the surgery.

**Surgical Procedures**

The surgery was performed randomly by a single experienced eye surgeon. The right eye pupil was dilated using phenylephrine hydrochloride of 2.5% and cyclopentolate hydrochloride of 1%. The rabbits were anesthetized with intramuscular injection of ketamine hydrochloride (35 mg/kg), xylazine (5 mg/kg), and topical anesthetic using 0.5% tetracaine hydrochloride.

The eyelids were disinfected with 10% povidone-iodine. The operating field was fixated on a sterile drape. The eyelids were opened with a lid speculum and disinfected with 5% povidone-iodine irrigated with BSS. Phacoemulsification ultrasound exposure was performed by the modified technique of Nemet et al. using a phacoemulsification machine (NIDEK CV 9000R).

A corneal incision in the superotemporal area was made with a 2.75 mm keratome blade. The phaco tip position was beveled up in the center of the anterior chamber without touching the endothelium and anterior lens capsule. The phacoemulsification used the following parameters: aspiration flow rate of 25 ml/min, 70% power, 90 cm bottle height, and burst mode panel. The power was switched on and off alternately every 10 seconds, lasting 5 minutes.

The corneal incision was closed with corneoscleral sutures with 10.0 nylon thread and checked for tightness. All these procedures were performed using an operating microscope visualization (Appasamy Operating Microscope Brilliant Advent). During observation after surgery, Levofloxacin 0.5% eye drops were given every three hours.

One day after surgery, CV and hexagonality of corneal endothelial were measured. Then, all rabbits were sacrificed with decapitation, and the eyes were enucleated. The cornea was released from the eyeball, then fixed with 10% buffered formaldehyde (pH 7.0) for histological examination.

**Histopathology**

Corneal specimens were made paraffin block and stained with SOD1 antibody (GTX13498, GeneTex Lab., China). The positive cells were stained as brown in the endothelial cell cytoplasm. The number of positive cells was assessed on serially numbered slides in a blinded manner, using a 400x objective lens of a light microscope (Olympus microscope Cx51) equipped with an Olympus camera using SIS software) by one pathologist. In two fields of view, a semi-quantitative method using immunoreactivity score (IRS) assessment.

**Statistical Analysis**

The statistic was analyzed using IBM SPSS Statistic version 26.0. Comparisons of SOD1 expression, CV changes, and hexagonality changes between the two groups were performed using an independent t-test for normally distributed data; alternatively, the Mann-Whitney test for non-normally distributed data. The correlation between SOD1 expression with CV and hexagonality expression was analyzed using the Pearson test for normally distributed data or the Spearman test for non-normally distributed data. The p-values <0.05 were considered statistically significant.

**RESULTS**

Phacoemulsification was performed in 16 rabbits. All the rabbits were survived, and no complications during the phacoemulsification procedure. Anterior segment examination before surgery was within normal limits. Endothelial morphology (CV and hexagonality) analysis was carried out three times and the mean value was...
day after the surgery (21.88 and 23.88, respectively, p>0.05). CV changes in the treatment group were lower than in the control group (6.33 and 6.46, respectively) but failed to reach statistical significance (p>0.05) (Table 2).

2. Hexagonality
There was no difference between the mean hexagonality of the control and treatment groups before the surgery (70.88 and 70.83, respectively, p>0.05). There was no difference between the mean CV of the control and treatment groups one day after the surgery (66.38 and 65.71, respectively, p>0.05). CV changes in the treatment group were lower than in the control group (5.96 and 6.42, respectively) but failed to reach statistical significance (p>0.05) (Table 3).

Expression of Superoxide Dismutase-1 (SOD1)
The SOD1 expression was examined by immunohistochemical staining using a SOD1 antibody. Positive cells were analyzed under a light microscope with 400 times magnification. The SOD1 expression in the treatment group showed weaker than in the control group (Figure 2). The mean of SOD1 expression of the treatment group (6.56 ± 1.37) was lower than the control group (8.25 ± 1.13). Statistical analysis using the Mann-Whitney test showed a significant difference between the control and treatment groups (p=0.024, p<0.05) (Table 2).

Correlation of SOD1 expression with Morphology Changes (CV and Hexagonality)
The distribution of SOD1 expression and the changes in corneal endothelial morphology (CV and hexagonality) were not normally distributed across all groups. Spearman test was used to assess the correlation between SOD1 expression and CV change, with p=0.188 (p>0.05). The correlation between SOD1 expression and hexagonality change was p=0.763 (p>0.05). These findings indicate no correlation between the expression of

Clinical Evaluation
Post-phacoemulsification handheld slit lamp examination showed corneal edema was worse in the control group than in the treatment group (Figure 1). There was no infection in either group.

Endothelial Morphological Changes
1. Coefficient of Variation (CV)
There was no difference between the mean CV of the control and treatment groups before the surgery (16.58 and 19.04, respectively, p>0.05). There was no difference between the mean CV of the control and treatment groups one

Table 1. CV and Hexagonality Distribution before Phacoemulsification Exposure.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Groups</th>
<th>Treatment Groups</th>
<th>p-value (independent t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Control Groups</td>
<td>Treatment Groups</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>16</td>
<td>16.58 ± 2.85</td>
<td>19.04 ± 4.60</td>
</tr>
<tr>
<td>Hexagonality</td>
<td>16</td>
<td>70.88 ± 5.74</td>
<td>70.83 ± 3.28</td>
</tr>
</tbody>
</table>

Table 2. Coefficient of Variation (CV) Distribution.

<table>
<thead>
<tr>
<th>Groups</th>
<th>CV Pre Phacoemulsification</th>
<th>CV Post Phacoemulsification</th>
<th>CV change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.58 ± 2.85</td>
<td>21.88 ± 7.22</td>
<td>6.46 ± 5.51</td>
</tr>
<tr>
<td>Treatment</td>
<td>19.04 ± 4.60</td>
<td>23.88 ± 8.85</td>
<td>6.33 ± 7.97</td>
</tr>
<tr>
<td>p-value</td>
<td>0.220</td>
<td>0.628</td>
<td>0.494b</td>
</tr>
</tbody>
</table>

mean ± SD; a = independent t-test; b = Mann-Whitney test

Figure 1. Anterior Segment Examination Post-Phacoemulsification in (A) Control and (B) Treatment group during 1st day postoperative.

Figure 2. SOD1 antibody IHC examination was seen with a light microscope with 400 times magnification in the control group (a and b) and the treatment group (c and d).
SOD1 with change in CV or hexagonality (Table 5).

DISCUSSION

SODs is an endogenous antioxidant that plays a vital role in preventing disorders caused by oxidative stress. SOD plays a role in oxidative signaling as the first response to increased reactive oxygen species (ROS) and functions as an anti-inflammatory. Copper/Zinc-Superoxide Dismutase (CuZn-SOD), also known as SOD1, is localized in the cytoplasm. SOD1 is the most expressed in all the eye tissue, including the cornea. The normal activity level of SOD is still unclear. Behndig et al. evaluated the distribution of the superoxide dismutase (SOD) enzyme in the human corneal layer. The results showed that SOD1 and SOD3 were found in the corneal epithelium, stroma, and endothelial layer. Cejkova et al. evaluated antioxidant activity at various ages of rabbits and found that the SOD activity was significantly higher in young adults than in young and old age.

SOD activity in body tissue can be used as a quantitative marker for clinical diagnosis. Several studies have shown that SOD activity is inversely related to ROS production. Donna et al. reported SOD activity significantly lower in the senile and diabetic cataract groups than in the normal group. Ozmen et al. reported CuZn-SOD activity was significantly lower in diabetic cataract groups than in senile and diabetic cataract groups. Wang et al. reported a decrease in SOD levels in aqueous humor and the cataract lenses as the thickness of the lens nucleus. Excessive accumulation of ROS causes the excessive use of SOD which triggers the conversion of soluble protein to an insoluble protein that causes lens opacification. Nurwasis et al. reported that the increase of intraocular pressure (IOP) followed by a decrease of IOP to normal level would increase the expression of SOD1. The increase in SOD aims to suppress the production of free radicals, so that oxidative stress does not occur and prevents apoptosis.

Decreased SOD activity was also found in hyperthermic conditions. There was an increase in mitochondrial metabolism, which caused increased ROS production, so SOD activity decreased due to being used to fight excess ROS. Wang et al. evaluated the effect of hyperthermia on platelet cells and found that hyperthermia induces ROS production. ROS production was significantly increased at 42°C for 3 hours. In addition, mitochondrial superoxide production also increased with increasing temperature. The decrease in MnSOD activity was caused by hyperthermia conditions that reduced the expression of MnSOD protein levels. An imbalance of ROS production and antioxidant activity results in oxidative stress and induces mitochondrial dysfunction.

Our results showed that intraocular hypothermia perfusion significantly affected SOD1 expression. The expression of SOD1 in the hypothermia group was significantly lower than in the room temperature group. This result is not in accordance with our hypothesis, in which SOD activity in hypothermic conditions should be higher than its activity in the room temperature group. This condition may associate with inhibiting enzyme expression due to a decrease in all metabolic processes in hypothermic conditions. Hendriks et al. evaluated the effect of perfusion at different temperatures on kidney mitochondrial function. The results showed a significant decrease in mitochondrial oxygen consumption and production of ROS (H₂O₂) decreased by about 50% at 4°C compared to 37°C. In hypothermic conditions, there is a decrease in all metabolic processes, causing hypometabolic conditions so that there is a decrease in mitochondrial activity that lowers oxygen consumption and results in decreased H₂O₂ production. However, the production of H₂O₂ in hypothermic conditions is still relatively high compared to the decrease in oxygen consumption and mitochondrial membrane potential. This may be caused by the decreased expression of Manganese Superoxide Dismutase (Mn-SOD). Mn-SOD was decreased in refrigerated HEK293 cells. The decrease in Mn-SOD could be caused by inhibiting transcription and translation of enzymes in hypothermic conditions.

Previous studies have reported that Endothelial Cell Density (ECD) is a parameter used to evaluate the cornea’s condition after phacoemulsification, but ECD could not reflect the corneal endothelial recovery process. Endothelial morphological changes are more sensitive to evaluating the wound
healing process due to trauma and are the best indicator to assess the stability of the corneal endothelial layer. The limited ability of endothelial cell regeneration causes the remaining cells to enlarge and migrate to fill the damaged endothelial space. Pardasani & Lohiya compared CV and hexagonality of endothelial cells before and after phacoemulsification. A significant increase in CV occurred one day after surgery, then decreased at the end of the four weeks, and at the 12 weeks, never reached baseline. Hexagonality significantly decreased one day after surgery, then slightly increased at four weeks and 12 weeks, never reached baseline. They concluded that CV and hexagonality changes after phacoemulsification occurred 1 day after surgery.

Several studies have been conducted to observe the effect of hypothermic perfusion on corneal endothelial morphology. Praveen et al. reported that a decrease in temperature of 10°C in the ocular tissue could reduce the metabolic activity of the cornea by as much as 50%. Wan et al. evaluated the effectiveness of hypothermic perfusion during phacoemulsification in hard nuclear cataracts. The percentage of hexagonality was significantly higher in the 4°C group than in the room temperature group at one day postoperatively. Jiang et al. observed the effect of hypothermia perfusion in the phacoemulsification of uveitis-associated cataracts and found that the mean percentage of hexagonality was higher in the 4°C group than the 24°C group at 7 days postoperatively but not statistically significant between the two groups.

Our results showed that intraocular hypothermic perfusion had no significant effect on changes in CV and hexagonality. This result does not follow our hypothesis, where the change in CV and hexagonality is lower in hypothermic conditions. Several possibilities cause insignificant changes in CV and hexagonality, namely the process of morphological changes have not occurred optimally. Budiman reported that CV changes occurred from one day after surgery, peaked at 1 week after surgery, and decreased until 3 months after surgery. Atas et al. reported that the percentage of hexagonality occurred significantly one day after surgery and improved within 14 days after surgery.

The measurement area for CV and hexagonality is only carried out in the central area of the cornea, so it can affect the measurement results. Kim et al. evaluated the process of endothelial cell recovery after cataract surgery by examining ECD and Endothelial Cell Area (ECA) in the central area and four paracentral areas (superior, inferior, temporal, and nasal) using a noncontact specular microscope. There was a significant increase in CV only in the temporal area at 1 day, 1 week, and 4 weeks after surgery. Significant damage in the temporal area is related to the position of the clear corneal incision in the temporal area and the movement of the phaco tip in and out of the incision.

Our results showed no correlation between SOD1 expression and changes in CV or hexagonality. In hypothermic conditions, there is a decrease in mitochondrial metabolism, which causes a decrease in antioxidant activity (SOD) and plays a role in fighting ROS. This is thought to result in relatively high endothelial damage, so there is no significant difference in changes in CV and hexagonality between the two groups.

In this study, hypothermic perfusion suppresses all metabolic processes, including the expression of SOD1 and ROS production. The coefficient of variation and hexagonality as a representative of corneal endothelial morphology changes also decrease with hypothermic perfusion after ultrasound exposure but are not statistically significant. There is no correlation between the expression of SOD1 and changes in CV and hexagonality.

There were several limitations in our study. We only examined the antioxidant activity of SOD1 by immunohistochemical staining and did not examine other antioxidants such as SOD2, SOD3, Catalase (CAT), and Glutathione Peroxidase (GPx). We did not evaluate ROS production directly to determine the direct effect of hypothermic perfusion on the corneal endothelium. We performed specular microscopy examinations only in the central area, while the paracentral areas, which may also have postoperative damage, were not examined. Further studies are required to examine other antioxidants’ activity and ROS production directly. Need a more precise method of examining the endothelial morphology (CV and hexagonality) that is not only central but on the entire surface of the cornea.

CONCLUSION

Hypothermic perfusion suppresses all metabolic processes, including the expression of SOD1 and ROS production. The coefficient of variation and hexagonality as a representative of corneal endothelial morphology changes also decrease with hypothermic perfusion after ultrasound exposure but are not statistically significant. There is no correlation between the expression of SOD1 and changes in CV and hexagonality.

ETHICAL CLEARANCE

The Ethics Committee has approved ethics approval for Basic and Clinical Science Research, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia (No: 2.KEH.048.04.2022).

CONFLICT OF INTEREST

There is no conflict of interest regarding the study.

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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.

REFERENCES


