The Effect of Temperature of Eye Irrigation Solution to Reduce Corneal Endothelial Cell Loss during Phacoemulsification: An *In Vitro* Model Study

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Objective: *To compare the efficiency of eye irrigation solution (balanced salt solution: BSS) stored in a refrigerator and air-conditioned room to reduce the number of corneal endothelial cell loss during the process of phacoemulsification.*
Material and Method: *Porcine corneal endothelial cells (PCEC) isolated from porcine eyes were used as a model. The porcine eyes were obtained from a local slaughterhouse within 6 h after death and PCEC were cultured as standard method. The percentage of cell survival was evaluated by MTT assay.*
Results: *The PCEC was successfully grown in the standard culturing system. The growth curve of PCEC demonstrated that log phase was reached in 3-6 days. The evaluation of heat effect (40-60°C) to PCEC survival was shown that cells death was found at 55 and 60°C which the percentage of cell survival reduced to 30% at 60°C, 180 sec. Further observation on the efficiency of BSS stored in different conditions, refrigeration (8°C) and air-conditioned room (25°C) to PCEC survival was revealed that the number of cell survival increased from 40% to almost 70% when using refrigerated BSS as irrigation solution, while from air-conditioned BSS only at 55%.*
Conclusion: *From using PCEC as a model and mimic the process of irrigation in phacoemulsification, it was found that the eye irrigation solution (balanced salt solution: BSS) stored in the refrigerator had a better result in helping protection the corneal endothelial cell loss from heat damaged than BSS stored in air-conditioned room.*

Keywords: *Corneal endothelial cells, Balanced salt solution, Heat, Phacoemulsification*

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Cataract is the clouding of the lens that blocks some of the light and make vision lost. Most cataracts are related to the natural aging of the eye. Other factors are possibly due to diseases such as diabetes, glaucoma or ultraviolet exposure. The symptoms of cataract may hard to detect at the beginning. The patient mostly aware when the vision is significantly lost. Treatment of cataract is only surgery, so far no medication or diet has been found to reduce this symptom. The technique that surgeon usually uses for removing the cataract is called phacoemulsification. This technique based on the utilization of ultrasonic power to break or emulsify the cataract lens into small pieces and after aspirated from the eyes the artificial lens is inserted. To remove the broken up eyes, the eye irrigation solution usually balanced salt solution is needed to replace in order to maintain the anterior chamber and also cooling the internal system. Generation of heat in the internal eye system comes from both the frequency and stroke length of the ultrasonic power used. The frequency is the stroke of needle movement which is usually between 20 and 50 kHz. Whereas the stroke length controlled by surgeon represents the actual distance the needle travels as it moves back and forth. Other parameters that may affect to the phaco power are aspiration flow and vacuum pressure[1]. If all parameters are not well controlled the risk of thermal injury is increased especially to the endothelial cells, the innermost layer of cornea that

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face to the anterior chamber. This corneal endothelial cell is significantly in consideration as it could not regenerate or divide after trauma or any insults[2,3]. The healing is done only by enlarging their monolayer cells rather than mitosis. Hence, the number of endothelial cell loss is important and must not less than the threshold for maintaining the function of lens transparency which is typically in the range of 500-1,000 cells/mm^2[4,5].

In the present study, the authors aim to investigate the effect of the eye irrigation solution using in the process of phacoemulsification to reduce the number of corneal endothelial cells loss. The irrigation solution was compared between being stored in a refrigerator and air-conditioned room that is usually used in the hospital. The porcine corneal endothelial cells were used as a model for studying due to the genetic is closely to human and convenience in obtaining. Besides, many reports have shown that porcine corneal endothelial cells are easy in achieving, maintaining and could be a good representative for human[6-8].

Material and Method

Materials

Porcine eyes were obtained from a local slaughterhouse within 6 h after death. Dulbecco's Modified Eagle's Medium (DMEM), Trypsin-EDTA, Pennicillin-streptomycin and Fetal bovine serum (FBS) were purchased from Gibco, Invitrogen, USA. (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrasoliumbromide (MTT) was purchased from Sigma, St.Louis, USA.

Methods

Porcine corneal endothelial cell (PCEC) isolation[6]

Porcine eyes were rinsed in distilled water twice before immersed in 70% ethanol for 2-3 min. After that, porcine eyes were rinsed with distilled water twice again. Then, corneas were dissected without scleral ring under sterile conditions by placing in a 60 mm tissue culture dish and washed several times with the medium (Dulbecco's Modified Eagle's Medium containing 100 UI/ml penicillin-streptomycin and 10% fetal bovine serum) and followed with phosphate buffer saline (PBS) twice. Next, 50 µl of Trypsin-EDTA was applied onto the endothelium and incubated at 37°C for 5 min to detach the cells. During the last minute, the endothelial cells (the inner layer of cornea) were transferred by a steriled spatula to a well (24-well plate) containing 2 ml of complete medium. To have another cell type control, the epithelial cells (the outer layer of cornea) were also extracted with the same way. The suspended cells were incubated in 5% CO_2 incubator at 37°C. Cells growth was evaluated every other day using an inverted microscope. Medium was changed every week until the cells were confluence. To measuring the growth curve, three independent experiments were performed which the cell number was counting by using trypan blue exclusion dye.

Subculture of PCEC

Confluent cells were reached in about 2-3 weeks. For sub-culturing, the old medium was discarded and cells were washed with PBS once and incubated in trypsin-EDTA for 1-2 min. After that, cells were washed with DMEM and centrifuged. The cell pellets were resuspended in the culture medium and seeded into T75 flasks which the medium was changed every other day. Sub-culturing was performed every the 2nd week at a split ratio of 1:3.

Effect of heat to PCEC survival

PCEC were seeded into a 24-well plate at a density of 2 x 10^4 cells per well in 1 ml DMEM and incubated at 37°C, 5% CO_2 for 48 h. Old medium was changed to a fresh one and cells were incubated further at 37°C, 5% CO_2 for at least 1 h. At each temperature tested (40, 45, 50, 55 and 60°C), cells were rapidly changed with the medium that already prepared by incubated in that temperature for at least 30 min before used. Each experiment was done by floating in a water bath that already set at the same temperature as the medium. The incubation times were set as time point at 0, 15, 30, 45, 60, 90, 120 and 180 seconds for each temperature tested. At the end of each time point, cells were changed with new culture medium and incubated at 37°C, 5% CO_2 for 2 h for cell adaptation. Three independent experiments were used in the present study.

Effect of BSS stored in different temperature in reducing the number of PCEC loss from heat damaged

Balanced salt solution (BSS) was stored in the refrigerator (8°C), air-conditioned room (25°C) and incubator (37°C) for at least 30 min before used. PCEC were heated at 60°C as described above. Every 15 sec, cells were changed between BSS and medium (60°C). Until 3 min, cells were changed with new culture medium and incubated at 37°C, 5% CO_2 for 2 h for cell adaptation.

Cell survival measurement

The tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrasoliumbromide (MTT) assay was used to evaluate the number of cell survival. Briefly, cells were incubated with MTT solution (5 mg/ml) for 2 h. After that, the formazan product occurring from the healthy cells were dissolved with dimethylsulfoxide (DMSO). The purple color of formazan was evaluated by spectrophotometer using the wave length of 540 nm. Each treatment was assayed in triplicate and at least three independent experiments were performed. The untreated cells were used as positive controls to evaluate as 100 % of cell survival.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Statistical significance between tested sample and control group was determined by using one way ANOVA analysis of variance. P-values < 0.05 were considered significantly different from the control group.

Results

In vitro culture of PCEC

Endothelial cells after extracted from corneas by trypsin-EDTA treatment were characterized as globular shape and suspended in the medium. After one day of incubation, the viable cells were attached to the plate surface, while the dead cells were floated in the medium. Within the first week, the number of the attached cells gradually increased detected by inverted microscope. The confluent cells were reached in about 2-3 weeks which most of them were appeared as hexagonal shape (Fig. 1). For cell type controlling, the isolation of epithelial cells was compared. However, most of them could not proliferate after 3 days of culturing and all were died in a week. The repetition of epithelial cells culturing was done several times but the results are similar. PCEC that suitable for testing was sub-culturing for at least 3 passages. And a growth curve was done as standard method which the result demonstrated that the log phase was reached in 3-6 days (Fig. 2).

Effect of heat to PCEC survival

To mimic the condition of heat generated in the process of phacoemulsification, a range of temperature (40-60°C) were selected for testing. The incubation of PCEC at each temperature was performed at 0, 15, 30, 45, 60, 90, 120 and 180 sec. As shown in Fig. 3, the number of cell survival was not reduced within 3 min when incubated at 40-50°C. However, at 55°C, the percentage of cell survival of PCEC was reduced to 80% and 40% at 120 and 180 sec respectively. In addition, the number of cell loss was significantly observed when incubated at 60°C which cell survival was reduced to 70% within 15 sec of incubation and gradually reduced to 30% in 180 sec.

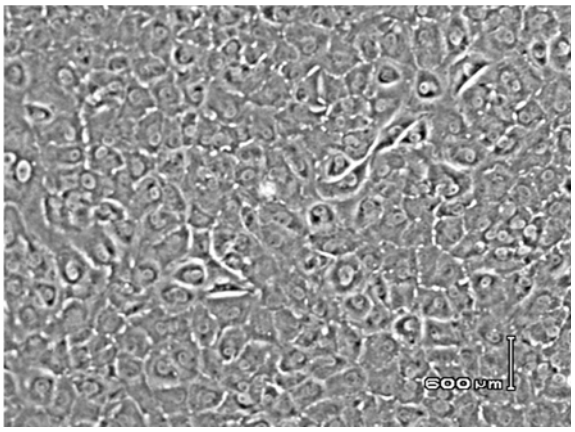

Fig. 1 The characteristic of porcine corneal endothelial cells (PCEC). PCEC was isolated from porcine eye and cultured in complete medium. About 2-3 weeks cells were confluence with hexagonal shape (magnification 200x)

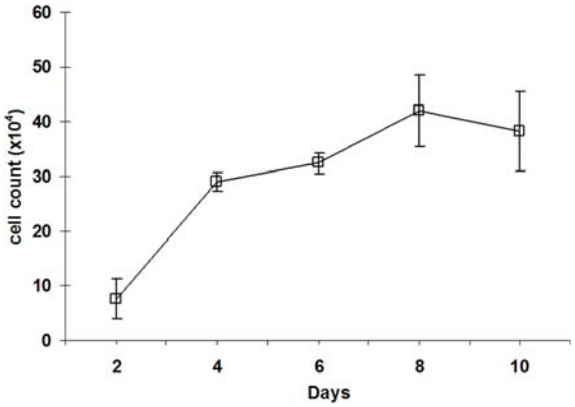

Fig. 2 Growth curve of PCEC. Cells were grown for 10 days in a standard cell culture system. Every two days, the number of cell was counted by using trypan blue exclusion dye. Results are mean ± SD from three independent experiments

Effect of different temperature BSS in reducing the number of PCEC loss from heat damaged

As the authors hypothesized that temperature of BSS may influence the number of endothelial cell

survival in phacoemulsification. Two conditions that usually used in the hospital were compared in the present study; storing in a refrigerator and air-conditioned room. The number of PCEC survival in each temperature BSS was tested prior to evaluating the efficiency in reducing the number of PCEC loss from heat damage. The data showed that either in the refrigerator or in an air-conditioned room, BSS itself was not harmful to the survival of cells (Fig. 4). Then, the effect of the temperature of BSS to reduce the number of PCEC loss during the process of phacoemulsification was performed. The condition of cell damaged at 60°C (about 40% cell survival) was selected as a model for evaluating. In addition, to mimic the process of phacoemulsification that needs to irrigate the eye intermittently, the authors designed the experiment by replacing the medium (60°C) of PCEC that causes cell damage with BSS every 15 sec. As depicted in Fig. 5, cells treated at 60°C had a large number of cell deaths which could be observed from the characteristic of cell shrinkage, rounding up and floating. The reaction when replacing with BSS the amount of cell death was reduced. It was also found that refrigerated BSS (8°C) gave the highest efficiency in protection comparing to BSS kept in an air-conditioned room (25°C) and in control incubator temperature (37°C). The number of cells survival was measured again from each reaction to confirm the data of the result which the result demonstrated that the refrigerated BSS was better in reducing cell loss (Fig. 6).

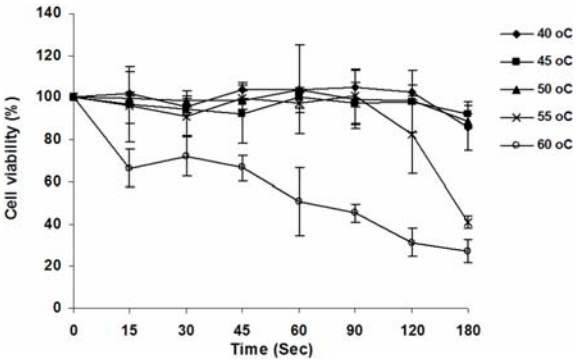

Fig. 3 Effect of temperature on the survival of PCEC. Cells were treated with BSS preincubated at 40, 45, 50, 55 and 60°C. At each temperature, the percentage of viable cells was calculated every 15 sec until 3 min using MTT assay. Results are mean ± SD from five independent experiments

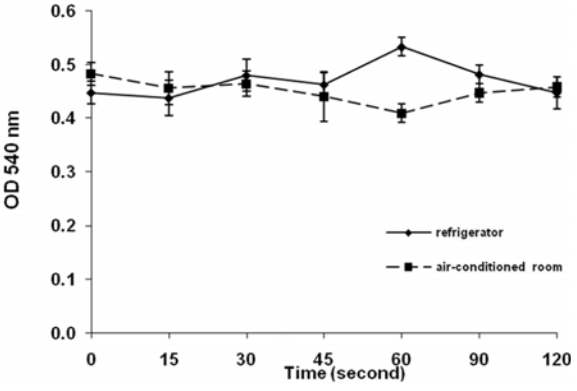

Fig. 4 The effect of refrigerated and air-conditioned BSS to PCEC survival. Cells were incubated either in refrigerated BSS or air-conditioned BSS for 120 sec. The number of cell survival was investigated by MTT assay and presented as the intensity of OD_{540}. Results are mean ± SD from three independent experiments

Discussion

Nowadays, phacoemulsification is recognized to be safer and faster than any other cataract surgery. However, several reports have shown that corneal endothelial cell loss still indicated[9-11]. In addition, transient postoperative corneal edema sometimes occurred after surgery[12,13]. These have been postulated that the procedure of phacoemulsification, especially the power of ultrasonic and duration used might be the cause of corneal endothelial cell damaged. The ultrasound could exert two kinds of influence on tissue: thermal effects and non-thermal effects[14]. Thermal effects are caused by the conversion of ultrasonic energy into thermal energy but in phacoemulsification it corresponds to thermal burn of cornea. Meanwhile, non-thermal effects represent acoustic cavitation and the resultant shock waves and formation of free radicals. A thermal burn can occur early in the process of phacoemulsification and causing damage to the surrounding tissue. The complications after surgery include delay wound healing, fistula formation, corneal stroma and endothelial damage, inability to close the incision and increased surgically induced astigmatism[15]. From finite element method (FEM), it has been shown that heat generation rate of phaco needle was 0.0004 cal/s/mm^2. Maximum temperatures of corneal endothelial were 52.67 and 41.57°C of two models tested at 60 sec[16]. In rabbit cornea model, it was shown that temperature up to 50°C for 10s was no evidence of histological damage. At 50°C, initial stromal collagen showed disorganization and damage of keratocyte. At 60°C time 0, massive corneal damage was found;

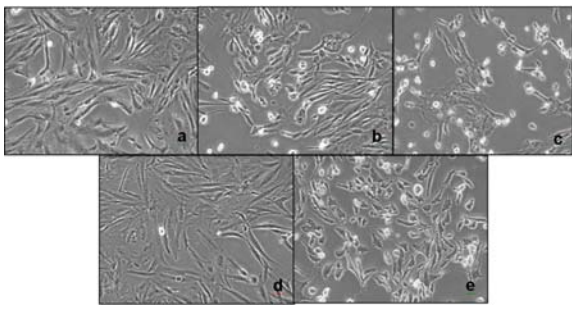

Fig. 5 The morphology of PCEC damage. The PCEC was heated at 60°C and replaced with a) refrigerated BSS (8°C), b) air-conditioned room BSS (25°C) and c) incubator BSS (37°C) every 15 min. The characteristic of cell death was shown as cell shrinkage, rounding up and floating. The control PCEC incubated in 37°C (d) and in 60°C of medium (e) was also shown

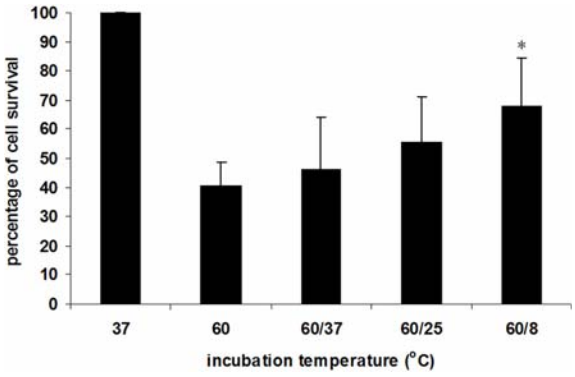

Fig. 6 The effect of different temperature of BSS in reducing the number of PCEC loss. Temperature of BSS was compared between refrigerated, air-conditioning room and 37°C as control. The condition of thermal damage at 60°C was used as a model. Each BSS was preincubated at least 30 min before replacing with the heated medium every 15 sec. The percentage of cell survival was determined by MTT assay. Results are mean ± SD from three independent experiments. Significant differences from control damaged cells (60°C) was indicated by *p < *0.05*

epithelial cell edema, collagen disorganization, severe stromal edema and endothelial cell detachment[17]. These findings agree with our results that the temperature at 40, 45 and 50°C was not harmful to the endothelial cells but at 55 and 60°C these cells were caused cell damage in 3 min. To prevent thermal damage, there are several factors that need to be concerned by surgeon, for example, time of phaco needle in the anterior chamber, turbulence of infusion fluid, viscoelastic agent and irrigation solution. BSS is a solution that is commonly used for irrigation as it is more closely to physiological condition including ionic strength, pH and osmolarity. The temperature of BSS has been shown that both normothermal and hypothermal do not affect postoperative parameters nor prevent burns[18]. However, in the present study it was found that BSS with low temperature could reduce endothelial cell loss comparing to normothermal (37°C). This might be due to the sensitivity of the method of this experiment which was done directly to the cell. The effect of refrigerated BSS to protect corneal endothelial cells was clearly seen as shown in Fig. 5. The characteristic of cell death was almost absent in the heated sample that replaced with refrigerated BSS. This data was confirmed when the number of cell death was counted (Fig. 6). Thus the benefit of hypothermal, refrigerated irrigation solution, to prevent thermal damage to corneal endothelial cells during phacoemulsification is suggested.

Conclusion

The BSS stored in the refrigerator has higher efficiency in cellular protection from heat damage than BSS stored in the air-conditioned room. This finding may be useful for eye surgeon to reduce the complication after surgery.

Acknowledgement

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Potential conflicts of interest

None.

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การศึกษาในหลอดทดลอง (in vitro) ถึงผลของอุณหภูมิของน้ำชะลูกตาในการลดการสูญเสียเซลล์เยื่อบุชั้นในของกระจกตาระหว่างการผ่าตัดต้อกระจก

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วัตถุประสงค์: *เพื่อเปรียบเทียบประสิทธิภาพของน้ำชะลูกตาชนิด balanced salt solution (BSS) ที่เก็บในตู้เย็นและในห้องปรับอากาศในการลดการสูญเสียเซลล์เยื่อบุชั้นในของกระจกตาที่เกิดขึ้นระหว่างการผ่าตัดต้อกระจก*

วัสดุและวิธีการ: *เซลล์เยื่อบุชั้นในของกระจกตาหมูได้เลือกมาใช้เป็นแม่แบบในการทดลอง เตรียมโดยการแยกจากลูกตาหมูที่ได้จากโรงฆ่าสัตว์ในระยะเวลาไม่เกิน 6 ชั่วโมง โดยเซลล์ที่แยกได้เลี้ยงด้วยวิธีมาตรฐาน สำหรับวิธีการหาร้อยละของเซลล์ที่รอดชีวิตใช้วิธี MTT assay*

ผลการศึกษา: *สามารถแยกและเพาะเลี้ยงเซลล์เยื่อบุชั้นในของกระจกตาได้โดยมีกราฟของการเจริญเติบโตที่แสดงให้เห็นว่าจำนวนของเซลล์จะถึงช่วง log phase ภายใน 3-6 วัน เซลล์ที่เตรียมได้นำมาทดสอบถึงผลของอุณหภูมิต่อการอยู่รอด ซึ่งอุณหภูมิที่ใช้ทดสอบคือ 40-60 องศาเซลเซียส และผลการทดสอบพบว่าที่อุณหภูมิ 55 และ 60 องศาเซลเซียสทำให้เซลล์ตาย และที่อุณหภูมิ 60 องศาเซลเซียสพบเซลล์ตายมากถึงร้อยละ 30 ที่เวลา180 วินาที นอกจากนั้นเมื่อเปรียบเทียบผลของอุณหภูมิของน้ำชะลูกตาที่เก็บในตู้เย็น (8 องศา) กับที่เก็บในห้องปรับอากาศ (25 องศา) ในการลดการสูญเสียเซลล์เยื่อบุชั้นในของกระจกตาพบว่าการใช้น้ำชะลูกตาที่เก็บในตู้เย็นจะช่วยลดการสูญเสียได้ดีกว่าโดยทำให้มีเซลล์ที่ยังมีชีวิตเพิ่มขึ้นจากร้อยละ 40 เป็นร้อยละ 70 ในขณะที่การใช้น้ำชะลูกตาที่เก็บไว้ในห้องปรับอากาศเพิ่มขึ้นเป็นร้อยละ 55 เท่านั้น*

สรุป: *จากการใช้เซลล์เยื่อบุชั้นในของลูกตาหมูเป็นแม่แบบในการทดลองและทำการทดสอบที่เลียนแบบการทำงานของน้ำชะลูกตาในการผ่าตัดต้อกระจก พบว่าน้ำชะลูกตาที่เก็บในตู้เย็นมีประสิทธิภาพในการลดการสูญเสียเซลล์เยื่อบุชั้นในของลูกตาได้ดีกว่าน้ำชะลูกตาที่เก็บในห้องปรับอากาศ โดยจำนวนของเซลล์ที่ได้รับความร้อนมีการสูญเสียที่ลดลงเมื่อใช้น้ำชะลูกตาที่เก็บในตู้เย็นในการทดสอบ*